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TOWNSEND and TOWNSEND and CREW LLP

By: Judith Cott

PATENT
Attorney Docket No.: 025663-000900US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Stephan R. Targan et al.

Application No.: 09/575,061

Filed: May 19, 2000

For: DIAGNOSIS, PREVENTION AND TREATMENT OF CROHN'S DISEASE USING THE OMPC ANTIGEN

Customer No.: 20350

Confirmation No. 1578

Examiner: Gailene Gabel

Technology Center/Art Unit:

DECLARATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Esther Oh, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true.

2. I am currently employed by Prometheus Laboratories Inc., the licensee of the subject application.

3. I am employed as a Product Development Manager and have been involved in the research and development of diagnostics for inflammatory bowel disease since 1999. My *Curriculum Vitae* is attached as Exhibit A.

4. I have reviewed and analyzed the above-referenced patent application, and I am familiar with the contents therein.

5. I have reviewed the contents of the Office Action dated September 15, 2005. In the Office Action, the Examiner alleges that claims 1-7 and 14-21 are not enabled for using solely IgA anti-OmpC antibody as a diagnostic marker for diagnosing the presence of Crohn's disease (CD). In view of the objective evidence set forth herein, the Examiner's concerns are overcome.

6. I have analyzed the ability of anti-OmpC antibodies to diagnose CD using samples from 185 CD and 127 control subjects (n=312). The samples were assayed for IgA antibodies to OmpC using an enzyme-linked immunosorbent assay (ELISA). As a comparison, the samples were also assayed for ASCA (*i.e.,* ASCA-A or ASCA-G), which represents an established marker for CD. For example, Ruemmele *et al.* (*Gastroenterology*, 115:822-829 (1998)), attached as Exhibit B, describes the use of ASCA (*i.e.,* ASCA-A and/or ASCA-G) as a highly specific diagnostic marker for CD. In addition, Vermeire *et al.* (*Gastroenterology*, 120:827-833 (2001)), attached as Exhibit C, describes three commercially available assays using ASCA (*i.e.,* ASCA-A and/or ASCA-G) as a highly specific marker for diagnosing CD.

7. Using a cutoff of 7.03 units/ml, the clinical performance of anti-OmpC antibodies (inventive) in diagnosing CD is shown below. The clinical performance of ASCA-A (comparative) and ASCA-G (comparative) in diagnosing CD using a cutoff of 6.16 and 11.45 units/ml, respectively, is also shown.

	Anti-OmpC (inventive)	ASCA-A (comparative)	ASCA-G (comparative)
Prevalence	59.3%		
Sensitivity	75.1%	75.1%	75.1%
Specificity	72.4%	79.5%	71.7%
Positive Predictive Value (PPV)	79.9%	84.2%	79.4%
Negative Predictive Value (NPV)	66.7%	68.7%	66.4%
Accuracy	74.0%	76.9%	73.7%
Area Under the ROC Curve	0.805	0.830	0.830

8. As shown above, the clinical performance of anti-OmpC antibodies is comparable to that of ASCA-A or ASCA-G in diagnosing CD. In fact, clinical parameters such as sensitivity, specificity, PPV, NPV, and accuracy are similar for all three markers. Furthermore, the similarity in the values for the area under the Receiver Operator Characteristic (ROC) curve (p-value >0.4 comparing curves) shows that all three markers possess comparable discriminatory power in differentiating between CD and controls. Since the use of either ASCA-A or ASCA-G alone is known in the art to be highly specific for diagnosing CD, it is my scientific opinion that the striking similarity in the ability of anti-OmpC antibodies (inventive) and ASCA-A (comparative) or ASCA-G (comparative) to diagnose CD indicates that anti-OmpC antibodies can also be used as a sole diagnostic marker for CD.

9. It is also my understanding that the European Patent Office has acknowledged the patentability of claims drawn to using solely IgA anti-OmpC antibody as a diagnostic marker for CD in corresponding European Patent Application No. 01935666.6, now European Patent No. 1285271. A copy of the issued patent is attached as Exhibit D.

10. In view of the foregoing objective evidence, it is my belief that claims 1-7 and 14-21 are enabled for using solely IgA anti-OmpC antibody as a diagnostic marker for diagnosing the presence of CD.

The declarant has nothing further to say.

Esther Oh

Esther Oh

02/14/06

Date

60668710 v1

Esther Oh
12461 Heatherton Ct. #310
San Diego, CA 92128
Email: scripsranch@gmail.com

Summary: Experienced in all phases of *in vitro*/clinical diagnostic product development from concept, product design/specifications, project development plan/timelines, reagent preparation, assay development and optimization, analytical validation, clinical studies to validate diagnostic utility, documentation, training and transfer to clinical (CLIA high complexity) laboratory operations; provide input to development of testing reports; provide support for new product launch.

Professional Experience
Prometheus Laboratories Inc., San Diego, CA
Manager, Product Development — June, 2000 - present
Sr. Research & Development Scientist — 1999

Responsibilities
- Develop new diagnostic assay products, determine analytical and clinical performance characteristics to validate assays (NCCLS Guidelines), write validation protocols and technical reports, clinical study protocols and data report forms, data/statistical analyses, preparation of materials (data tables, graphs, charts) for scientific abstracts, peer-reviewed manuscripts/publications, presentations and patent submissions, collaborate with investigators/consultants for technology assessment and transfer, and development and conduct of clinical studies.
- Evaluate and implement new technology platforms to improve efficiency, throughput and performance of current assays
- Identify, evaluate, select and validate commercial assay kits for use in clinical laboratory
- Manage projects to ensure adherence to product launch timelines; provide support to Marketing and Operations through new product launches
- Maintain scientific expertise by monitoring and surveillance of current scientific literature, publications, patents, new technology and competitive commercial products
- Supervise team of research associates

Accomplishments:
- Developed and validated non-invasive panel of three serum markers (Fibrospect™, a regression algorithm) to differentiate mild from more severe liver fibrosis in chronic HCV patients (including clinical studies to validate diagnostic performance)
- Developed and validated product for HLA typing of celiac disease associated alleles (by PCR-SSP) for clinical laboratory
- Developed and validated immunoassays to quantitate levels of chimeric antibody drug and drug-specific human anti-chimeric antibodies, and transferred to clinical laboratory practice
- Completed clinical studies and data analyses in support of validation of assay products
- Evaluated several recently discovered (recombinant) protein antigens as potential targets for new diagnostic immunoassays; developed and validated improvement to 4-marker serology test panel (immunoassays) optimized for maximum diagnostic sensitivity and specificity
- Provided scientific and technical evaluations of various technologies under consideration by Business Development for licensing

Amylin Pharmaceuticals Inc., San Diego, CA
Sr. Research Associate, Preclinical Development — 1997-98

Accomplishments:
- Developed and validated immunoassays for quantitation of peptide drugs in pharmacokinetic and clinical samples; analyzed data, wrote documentation, research and validation reports.
- Determined stability of drug in plasma matrix, and stability of standards and controls.
- Researched and evaluated enzymatic (colorimetric and fluorescence substrates) and other signal amplification detection systems to improve assay sensitivity.
- Supervised research associates.
- Knowledge of cGLP/cGMP.

Telios Pharmaceuticals, Inc., San Diego, CA
Product Manager, Wound Healing — 1989
- Established process development procedures and documentation, evaluated equipment and facilities for pilot scale production of peptide-based wound healing product.

Hybritech Incorporated, San Diego, CA

Research Scientist 1986-1988

- Developed rapid test for HIV1 antibodies and transferred to pilot plant
- Completed in-house preclinical testing and data analysis
- Designed and completed field evaluations of kit
- Developed QC and in-process tests
- Evaluated stability of kit and components
- Supervised 4 research assistants/associates

Senior Research Associate 1985

- Developed immunoassays to screen human monoclonal antibodies to tumor antigens
- Characterized tumor associated antigens recognized by human and mouse monoclonal antibodies

University of California, Department of Medicine, San Diego, CA

Assistant Specialist 1983-84

Staff Research Associate 1982

- Developed monoclonal antibodies to lymphocytes and platelets for characterization of autoantibodies in autoimmune disease (GP1b, GPIIb/IIIa)
- Developed immunoassays for detection of autoantibodies

Education

Specialized Certificate in Clinical Trials Design and Management, University of California San Diego Extension

M.Sc., Queen Elizabeth College, University of London, England

B.Sc. (Honours) in Biochemistry, University of Malaya, Kuala Lumpur, Malaysia

Oral Presentation

Clinical performance of two serological antibody panels for the diagnosis of IBD and the differentiation of CD from UC. DDW (American Gastroenterological Association Annual Scientific Meeting) Research Forum, May 14-17, 2005

Selected Publications

1. Diagnostic accuracy of a fibrosis serum panel FIBRO*Spect* II compared to Knodell and Ishak liver biopsy scores in hepatitis C patients. Carol Christensen, Dana Bruden, Stephen Livingston, Heike Deubner, Chris Homan, Katie Smith, Esther Oh, Dan Sullivan, David Gretch, James Williams, Brian McMahon. J Viral Hep (in press).
2. Evaluation of panel of non-invasive serum markers to differentiate mild from moderate-to-advanced liver fibrosis in chronic hepatitis C patients. Keyur Patel, Stuart C Gordon, Ira Jacobson, Christophe Hezode, Esther Oh, Katie Smith, Jean-Michel Pawlotsky, John G McHutchison. J Hepatology 2004; 41:935-942.
3. An improved biomarker panel (FIBRO*Spect*SM II) for differentiating mild from more severe liver fibrosis in chronic HCV patients. Oh E, Smith K, Patel K, Gordon SR, Pawlotsky J-M, Jacobson IM and McHutchison JM. Gastroenterology 2004; 126(4):S1639.
4. Highly specific PCR-SSP typing of celiac disease HLA DQ alleles. E Oh and M Barry. American College of Gastroenterology Annual Scientific Meeting, 2003. (Presidential Poster award).
5. Evaluation and optimization of a panel of serum markers for liver fibrosis in chronic hepatitis C patients. K Patel, J McHutchison, E Oh, P Nguyen and S Rose. Gastroenterology 2002; 122(4):M1610.
6. A non-invasive panel of serum markers can reliably differentiate hepatitis C patients with minimal fibrosis from those with fibrosis stages F2-F4. Patel K, Gordon SC, Oh E, Smith K and McHutchison JG. Hepatology 2002; 36(4 pt 2):355A
7. Optimization of four-marker serology diagnostic system for Inflammatory Bowel Disease. E. Oh, C. LaCerte and S. Rose. Poster presented at American Gastroenterological Association, Digestive Diseases Week, May 2001.
8. Quantitation of Remicade™ and Remicade-specific human anti-chimeric antibody (HACA) in Crohn's Disease patients. E. Oh, C. LaCerte, H. Thomsen and S. Rose. Poster presented at American College of Gastroenterology, 64th Annual Scientific Meeting, 1999.
9. A breast cancer targeted in vivo by a human monoclonal antibody. E. Oh, K. Burnett and J. Leung. (meeting abstract). Fed Proc 45(4): 984 (1986).
10. Autoantibodies against the platelet glycoprotein IIb/IIIa complexes in patients with chronic Immune Thrombocytopenic Purpura. V.L. Woods, Jr., E.H. Oh, D. Mason and R. McMillan. Blood 63:368-375 (1984).
11. Deposition of plasma fibronectin in tissues. E. Oh, M. Pierschbacher and E. Ruoslahti. Proc Natl Acad Sci 78:3216-3221 (1981).

GASTROENTEROLOGY 1998;115:822–829

Diagnostic Accuracy of Serological Assays in Pediatric Inflammatory Bowel Disease

FRANK M. RUEMMELE,* STEPHAN R. TARGAN,‡ GALITH LEVY,* MARLA DUBINSKY,* JONATHAN BRAUN,§ and ERNEST G. SEIDMAN*

*Division of Gastroenterology-Nutrition, Department of Pediatrics, Ste-Justine Hospital, University of Montreal, Montreal, Quebec, Canada; ‡IBD Center, Cedars Sinai Medical Center, Los Angeles, California; and §Department of Pathology, UCLA School of Medicine, Los Angeles, California

See editorial on page 1006.

Background & Aims: Accurate serological assays are desirable for the diagnosis of inflammatory bowel disease (IBD) types in the pediatric age group. The aim of this study was to test the diagnostic accuracy of modified assays for perinuclear (p) antineutrophil cytoplasmic antibodies (ANCAs) and anti-*Saccharomyces cerevisiae* antibodies (ASCAs) in patients with pediatric ulcerative colitis (UC) and Crohn's disease (CD) and in those without IBD. **Methods:** With observers blinded to patients' diagnoses, serum specimens were analyzed for immunoglobulin (Ig) A and IgG ASCAs and ANCAs by enzyme-linked immunosorbent assay. The perinuclear location of ANCAs visualized by indirect immunofluorescence was confirmed by its disappearance after administration of deoxyribonuclease. **Results:** IgA and IgG ASCA titers were significantly greater and highly specific for CD (95% for either, 100% if both positive). pANCA was 92% specific for UC and absent in all non-IBD controls. The majority of patients with CD positive for pANCA had a UC-like presentation. Disease location, duration, activity, complications, and treatment with immunosuppressive drugs did not have an impact on the ASCA or pANCA assay results. After resection, UC patients remained pANCA positive, in contrast to patients with CD, in whom ASCA titers decreased toward normal values postoperatively. **Conclusions:** ASCA and pANCA assays are highly disease specific for CD and UC, respectively. These serological tests can assist clinicians in diagnosing and categorizing patients with IBD and may be useful in making therapeutic decisions.

As in adults, the clinical presentation of inflammatory bowel disease (IBD) in children depends largely on the site and extent of the mucosal inflammation. However, the diagnosis of IBD is more often delayed in children because of the frequency of nonspecific symptoms at the onset of disease.[1] Reliable serological screening tests would potentially be helpful in identifying children and adolescents with IBD, resulting in earlier diagnoses.

Crohn's disease (CD) and ulcerative colitis (UC) are generally considered to be distinct forms of IBD. Yet, their symptoms and clinical presentations commonly overlap, and their discrimination in cases limited to the large bowel may be problematic.[2] Among patients with CD, a defined subgroup with a UC-like presentation has been described, illustrating the similarities of these diseases.[3] To distinguish these entities from each other and especially from self-limited intestinal inflammation, various clinical, radiological, endoscopic, and histopathologic criteria have been put forth. Nevertheless, a certain proportion of cases (10%–15%) defy clear categorization even after colectomy and are commonly referred to as indeterminate colitis (IC).[1,2,4] In the pediatric literature, IC is the term applied to cases of IBD limited to the colon with features suggestive of both UC and CD, without restricting the definition to patients who have undergone colectomy.[4] Furthermore, a diagnosis in a patient initially identified as having UC may, over time, be "switched" to CD in view of the extension of disease.[5,6] Accurate, noninvasive tests to distinguish such cases would be very helpful in such circumstances.

In UC, perinuclear (p) antineutrophil cytoplasmic antibodies (ANCAs) have been reported to be present in 60%–80% of cases, with a high degree of disease specificity.[7,8] Main et al. first identified increased titers of anti-*Saccharomyces cerevisiae* antibodies (ASCAs) in the sera of adult patients with CD compared with those with UC or healthy controls.[9] These antibodies are directed against distinct oligomannosidic epitopes of this yeast, as recently characterized by Sendid et al.[10] Recent technical

Abbreviations used in this paper: ANCA, antineutrophil cytoplasmic antibody; ASCA, anti-*Saccharomyces cerevisiae* antibody; CD-DX-1, Crohn's disease diagnostic system; DNase, deoxyribonuclease; ELISA, enzyme-linked immunosorbent assay; EU, ELISA unit; IC, indeterminate colitis; p, perinuclear; UC-DX-1, ulcerative colitis diagnostic system.

0016-5085/98/$3.00

advances in these assays have allowed us to enhance the disease-specific accuracy of the ASCA and pANCA diagnostic systems.[11]

This study was designed to test the diagnostic accuracy of these noninvasive tests, with special regard to their ability to discriminate pediatric patients with CD and colitis from those with UC and from non-IBD controls. We also examined the effect of disease location, duration, activity, complications, and medical as well as surgical treatments on ASCA and pANCA assay results.

Materials and Methods

Study Population

Serum was collected from pediatric patients attending the Ste-Justine Hospital gastroenterology inpatient service and IBD clinics. The selection of patients for this study was made on the basis of an absolute diagnosis in well-defined ("reagent-grade") patients (n = 173) who had undergone a complete diagnostic work-up for IBD. Furthermore, serum was collected from 36 consecutive patients who were prospectively assessed to rule out IBD. In all, 252 specimens from 209 consenting subjects were collected. All serum samples were coded so that analyses could be performed with the experimenter blinded to the patients' diagnoses. The study protocol was approved by the Ste-Justine Hospital Ethics Committee.

Clinical information for each patient was collected by chart review independently by two investigators unaware of the results of the antibody profiles. According to their concordant diagnosis established by standard clinical criteria and with endoscopic, histopathologic, and radiographic confirmation,[4] the patients with IBD were classified as CD (n = 130) or UC (n = 35). A third group consisted of patients (n = 11) whose initial endoscopic and histopathologic diagnosis was that of an indeterminate colitis (IC), based on the criteria of Chong et al.[12] Among these, further investigations over a period of 0.5–4 years led to a diagnosis of CD and UC (n = 1 case each); these patients were thus included in their respective CD and UC groups. The remaining 9 patients constituted our IC group. The control group (n = 78) without evidence of IBD consisted of patients with untreated celiac disease (n = 25), eosinophilic colitis (n = 16), irritable bowel syndrome (n = 16), acute self-limited bacterial colitis (n = 10), peptic ulcer disease (n = 3), autoimmune enteropathy (n = 2), gastroesophagal reflux (n = 2), or antral stenosis, intestinal pseudo-obstruction, polyposis, and an ischiorectal fistula (n = 1 each). Further characterization of these patient groups is shown in Table 1. Disease activity for the group with CD was calculated by using a modified Harvey–Bradshaw index[13] and by the Truelove and Witts scale for UC.[14]

CD Diagnostic System

The presence of ASCA was determined by a fixed enzyme-linked immunosorbent assay (ELISA; Prometheus Laboratory, San Diego, CA). Briefly, microtiter plates were coated with phosphopeptidomannans from the yeast *Saccharomyces uvarum*. Control and coded samples were added at a 1:100 dilution. Bound antibodies were labeled by alkaline phosphatase–conjugated goat anti-human immunoglobulin (Ig) G. After the addition of *p*-nitrophenol, specific absorbance was measured at 405 nm. The absorbance of each sample was evaluated and assigned ELISA unit (EU) values relative to the absorbance of a pool of sera collected from well-characterized patients with CD. The standard pool was arbitrarily assigned the value of 100 EU/mL. Results of the CD diagnostic system (CD-DX-1) panel were positive if either IgG or IgA or both were positive. Before this study, the cutoff for positivity was determined at Prometheus Laboratory on the basis of results in well-defined patients with CD and was set at 20 and 40 EU/mL for IgA and IgG ASCA, respectively.

UC Diagnostic System

The presence of ANCA was first screened for by means of a fixed neutrophil ELISA (Prometheus Laboratory) as described previously.[15] Briefly, microtiter plates were coated

Table 1. Characterization of the Patient Groups Studied

	CD		UC			
	CD-DX-1$^+$	CD-DX-1$^-$	UC-DX-1$^+$	UC-DX-1$^-$	IC	Non-IBD
n	71	59	20	15	9	78
Mean age (*yr*)	14.3 (10–21)	13.8 (1–21)	13.6 (5–17)	12.1 (8–18)	13.8 (8–17)	6.5 (0.1–20)
Disease duration (*mo*)	22.4 (1–96)	22.0 (0–70)	15.7 (0–52)	19.8 (2–54)	15.3 (10–52)	—
Active disease	11	10	2	0	1	—
Small bowel involvement	19	15	—	—	0	—
Large bowel involvement	8	11	20	15	9	—
Small and large bowel involvement	44	33	—	—	0	—
Complications	16	6	3	4	2	0
Surgery	4	7	3	2	1	0
Corticosteroids	37	31	5	6	5	0
6-Mercaptopurine	8	4	0	0	0	0

NOTE. In CD, a Harvey–Bradshaw index of >5 was defined as active disease; disease activity in children with UC was determined by means of the Truelove index. As noted in Materials and Methods, disease location was based on radiological, histological, and endoscopic findings. Complications included fistulae, perforation, stenosis, or obstruction in children with CD, or arthritis, pericarditis, or autoimmune thyroiditis in children with UC.

+, positive; −, negative.

with 2.5×10^5 methanol-fixed neutrophils per well. After nonspecific antibody binding was blocked with 0.25% bovine serum albumin, control and coded sera were added at 1:100 dilutions. Neutrophil-bound antibody was labeled by alkaline phosphatase–conjugated goat anti-human IgG. After the addition of *p*-nitrophenol, specific absorbance was measured at 405 nm. Before this study, the cutoff for positivity was determined by positive controls from well-defined patients with UC (mean, 13.9 EU/mL) at Prometheus Laboratory.

Indirect immunofluorescence staining was then performed on ANCA ELISA–positive samples to determine whether a predominantly perinuclear (pANCA) or cytoplasmic (cANCA) staining pattern was present. Briefly, methanol-fixed neutrophils on glass slides were incubated with the coded samples (1:20 dilution). Specific binding was visualized by fluorescence microscopy after the addition of fluorescein-labeled anti-human IgG. The specificity of the perinuclear staining pattern in UC was finally confirmed by its disappearance after deoxyribonuclease (DNase) treatment of the neutrophils (Prometheus Laboratory). Results of the UC diagnostic system (UC-DX-1) panel were considered positive when both the ANCA titer was above the cutoff and the indirect immunofluorescence revealed a perinuclear binding of ANCA that disappeared after DNase treatment.

Statistical Analysis

All variables were tested for normal distribution by means of the David, Pearson, and Stephens test. When necessary, a log transformation was performed to obtain a normal distribution. Analysis of variance was used for comparison of IgA ASCA, IgG ASCA, and ANCA titers between groups. Adjustment for multiple comparisons was made using the Bonferoni correction. χ^2 tests were used to compare qualitative variables between groups. A *P* value of <0.05 was considered significant.

Results

CD-DX-1 Assay System

Comparison of ELISA results showed that the mean levels for both IgA and IgG ASCA (Figures 1 and 2)

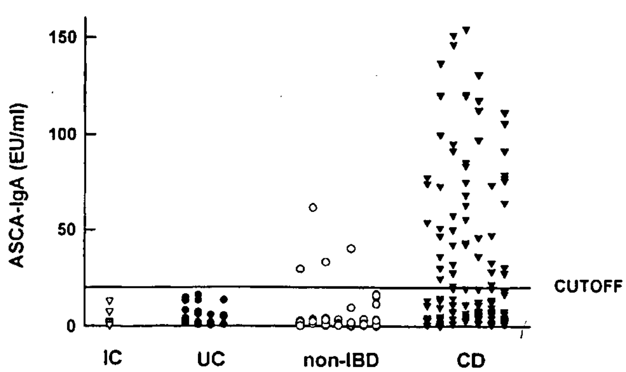

Figure 1. Scattergram showing IgA ASCA values as determined by ELISA in children with CD vs. children with UC, IC, and non-IBD controls.

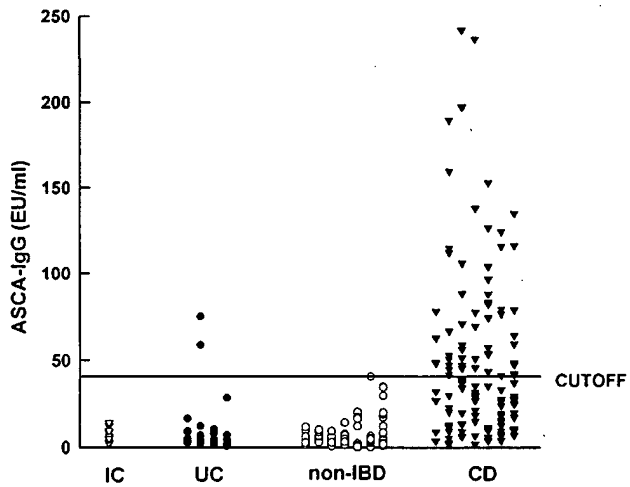

Figure 2. Scattergram showing IgG ASCA values as determined by ELISA in children with CD vs. children with UC, IC, and non-IBD controls.

were markedly higher in CD than the UC and non-IBD groups ($P < 0.001$). Moreover, as shown in Tables 2–4, the CD-DX-1 assay system (ASCA IgA and/or IgG) proved to be highly specific (95%) for CD, with 71 of 130 (55%) patients with CD being positive, compared with only 2 of 35 (6%) with UC, and 4 of 78 (5%) without IBD ($P < 0.005$). The combination of IgA and IgG ASCA positivity was 100% specific for CD. Only 1 of 11 patients initially classified as IC had a positive IgA ASCA titer. In the follow-up on this particular patient, a definitive diagnosis of CD was made 4 years after the initial blood sample was taken. Therefore, this patient was included in the CD group. None of the untreated celiac patients was ASCA positive.

UC-DX-1 Assay System

In distinct contrast, ELISA ANCA levels were significantly elevated in UC (Figure 3). Using indirect immunofluorescence with DNase, this assay system was highly specific (92%) for UC (Tables 2–4). Among patients with UC, 20 of 35 (57%) had a positive pattern compared with 17 of 130 (13%) and 0 of 78 (0%) children in the CD and non-IBD groups, respectively ($P < 0.005$). Antineutrophil cytoplasmic antibodies were present in some children in the CD and non-IBD groups. However, the perinuclear location characteristic of UC was not found in any non-IBD patients. Among the IC group, the 1 patient whose follow-up led to the diagnosis of UC was pANCA positive. Of the remaining 9 patients with IC, 6 had ANCA titers above the cutoff for positivity (Table 2). In 2 of these patients, the immunofluorescence after DNase treatment showed a true perinuclear straining pattern. Among the 17 patients with CD who were pANCA positive, 10 (59%) presented characteristic

Table 2. Results of ASCA and ANCA Assays in Pediatric Patients Evaluated for Possible IBD

Patient group	n	IgA ASCA+ n (%)	IgG ASCA+ n (%)	IgA or IgG ASCA+ n (%)	IgA and IgG ASCA+ n (%)	ANCA ELISA+ n (%)	pANCA indirect immunofluorescence after DNase n (%)
CD (all)	130	56 (43)	56 (43)	71 (55)	41 (32)	54 (42)	17 (13)
CD (colitis)	17	8 (47)	5 (29)	8 (47)	5 (29)	11 (65)	2 (12)
UC	35	0	2 (6)	2 (6)	0	31 (89)	20 (57)
IC	9	0	0	0	0	6 (67)	2 (22)
Non-IBD controls	78	4 (5)	0	4 (5)	0	8 (10)	0

+, positive.

UC-like symptoms, as recently defined by Vasiliauskas et al.[3] This subgroup comprised 8 CD patients with pancolitis and 2 others with continuous, left-sided colitis. Interestingly, 7 of the 8 (88%) CD patients with pancolitis had very high ANCA titers, similar to that observed in the UC group (68.2 vs. 57.7 EU/mL, respectively). However, ANCA titers were significantly ($P < 0.001$) higher for the UC patients compared with the entire CD colitis subgroup. Furthermore, 5 of these 8 patients with UC-like pancolitis CD had a clear perinuclear ANCA immunofluorescent pattern.

Disease Location

As shown in Tables 1 and 2, no relationship was found between CD-DX-1 assay positivity (either IgA or IgG ASCA or both) and the site of CD inflammation (small vs. large bowel involvement, or both; $P = 0.7$). Comparing untreated celiac disease patients as small bowel inflammatory controls confirmed that the highly significant increase for both IgA ASCA ($P < 0.01$) and IgG ASCA ($P < 0.0001$) was restricted to the CD group. Patients with CD limited to the colon tended to have lower IgA and IgG ASCA compared with others with CD ($P = 0.3$; Figures 4 and 5), although their levels were significantly higher ($P < 0.005$) compared with all other patients with colitis (UC, IC, eosinophilic and acute, self-limited colitis). The CD-DX-1 assay was highly specific for the CD colitis subgroup (Table 4): 96% with one ASCA positive and 100% with IgA and IgG ASCA positive (CD colitis vs. UC and non-IBD colitis). The UC-DX-1 assay was positive in 22% of patients with IC vs. 57% of patients with UC. Only 13% of the entire CD group (small and/or large bowel disease) and 12% of the CD colitis subgroup had a positive pANCA fluorescence pattern, confirmed by its disappearance after DNase treatment.

Disease Duration

No significant correlation was observed between the duration of symptoms in CD and the results of the CD-DX-1 assay (Table 1), excluding the 11 patients who had undergone surgery (22.9 months [range, 1–96 months] vs. 17.0 months [range, 0–70 months] for CD-DX-1–positive vs. –negative patients with CD; $P = 0.25$). Among children with a recent diagnosis of CD, 10 of 14 (71%) were positive for CD-DX-1. The results of this subgroup, tested during initial diagnostic workup, were not statistically different from those of the entire CD group ($0.5 < P < 0.7$). Among the 20 patients with CD who underwent repeated assays, 3 converted from ASCA negative to positive after a variable period (1 month to 5 years). Three others remained

Table 3. Diagnostic Accuracy of the Serological Tests to Distinguish Between CD, UC, and Non-IBD

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
IBD vs. non-IBD: CD-DX-1 or UC-DX-1 assay	55	95	96	50
CD vs. UC + non-IBD: CD-DX-1 assay (IgA *or* IgG)	55	95	92	64
CD vs. UC + non-IBD: CD-DX-1 assay (IgA *and* IgG)	32	100	100	56
UC vs. CD + non-IBD: ANCA ELISA	89	70	33	97
UC vs. CD + non-IBD: UC-DX-1 assay	57	92	54	93

Table 4. Diagnostic Accuracy of the Serological Tests to Distinguish Between CD Colitis, UC, and Non-IBD Colitides

	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
CD colitis vs. UC + non-IBD colitis: CD-DX-1 IgA *or* IgG ASCA	47	96	73	87
CD colitis vs. UC + non-IBD colitis: CD-DX-1 IgA *and* IgG ASCA	29	100	100	87
UC versus CD colitis + non-IBD colitis: ANCA ELISA	89	78	70	92
UC vs. CD colitis + non-IBD colitis: UC-DX-1 assay	57	97	91	79

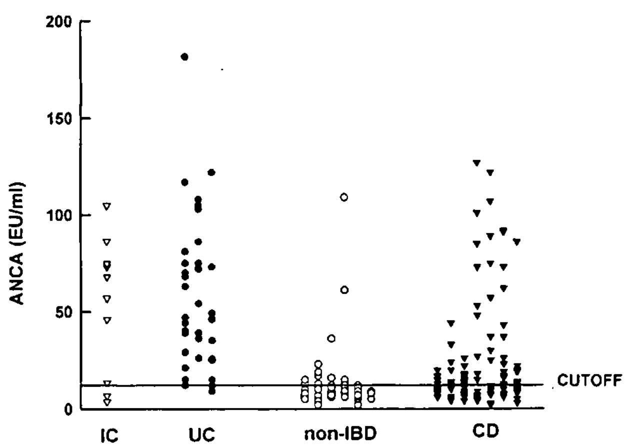

Figure 3. Scattergram showing ANCA titers as determined by ELISA in pediatric patients with UC, IC, and CD, and non-IBD controls.

CD-DX-1 negative; the others showed little variation (<25%) in ASCA titers over time. None of the initially ASCA-positive patients with CD converted to negative.

Similarly, disease duration did not influence the results of the UC-DX-1 assay system in patients with ulcerative colitis (mean, 15.7 and 19.8 months for UC-DX-1–positive and –negative groups, respectively; $P > 0.25$).

Disease Activity

We did not observe a statistically significant effect of disease activity on ASCA titers in the CD group or on ANCA results in the UC group. Among the patients with CD with active disease (n = 21/119, 18%), defined by a Harvey–Bradshaw index of >5, mean ASCA IgG was 82 EU/mL vs. 66 EU/mL, and mean ASCA IgA was 28 EU/mL vs. 34 EU/mL for the subgroup in remission (P = NS). ASCA double positivity in CD did not correlate with higher disease activity.

Complications

Serum IgA and IgG ASCA titers were not different among CD patients with complications in the form of

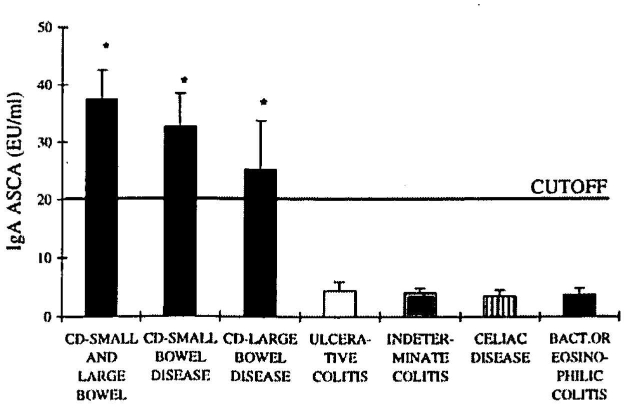

Figure 4. Effect of the site of inflammation on IgA ASCA titers (mean of each group ± SD). *$P < 0.01$, CD vs. UC or non-IBD.

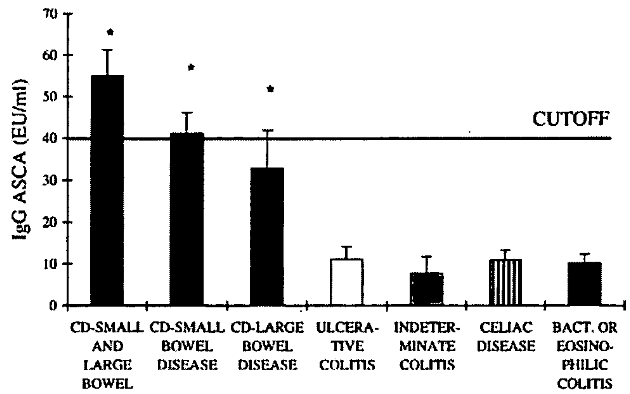

Figure 5. Effect of the site of inflammation on IgG ASCA titers (mean of each group ± SD). *$P < 0.01$, CD vs. UC or non-IBD.

fistulae, perforation, stenosis, or obstruction (n = 22, 18%) compared with those without such complications (Table 1). Among the patients with UC, 7 presented with extraintestinal complications, including arthralgias (n = 5), pericarditis (n = 1), and autoimmune thyroiditis (n = 1). These associated autoimmune phenomena did not have an impact on the results of the UC-DX-1 assay.

Medical Therapy

The use of systemic corticosteroids (intravenous or oral) in CD (n = 68, 52%) did not influence the results of the CD-DX-1-assay; their use in UC (n = 11, 31%) also had no effect on the results of the UC-DX-1 assay (Table 1). Similarly, mesalamine or 6-mercaptopurine (n = 12) use did not have an impact on these results compared with untreated findings in patients with IBD.

Surgery

Among patients with CD, 11 of 130 (8%) had undergone a resection. In 7 of these, the postoperative CD-DX-1 assay results were negative. The mean interval between surgery and testing was 3 years in the CD-DX-1–negative group, whereas the 4 patients positive for ASCA were all analyzed immediately postoperatively (<4 days). Unfortunately, these patients were not part of a prospectively conducted study, and therefore no serum from before surgery was available for comparison. In the group with UC, 5 of 35 (14%) had undergone surgery. Except for 1 child with negative results of an ANCA panel despite high ANCA ELISA titers, the 4 others remained pANCA positive after colectomy (mean interval between surgery and the assay, 4 months; range, 1–18 months).

Discussion

A dramatic increase in the incidence of CD during childhood has been recognized over the past few decades,

with a trend toward an increasing number under the age of 10 years at the time of diagnosis.[16-20] Prompt recognition of IBD, whose incidence peaks in the second decade of life, has thus become increasingly important for physicians caring for children and adolescents. In this study, we show for the first time that the CD-DX-1 and UC-DX-1 assay systems are highly specific diagnostic tools in pediatric CD and UC, respectively. As shown in Table 3, the specificity and positive predictive values of the two assays in discriminating IBD vs. non-IBD patients were 95% and 96%, respectively. Furthermore, only children with CD were double-positive for IgA and IgG ASCA (100% specific). The combination of ANCA testing by ELISA followed by immunofluorescence and the additional confirmatory step using DNase treatment of neutrophils with bound antibodies, resulting in the disappearance of the perinuclear staining, increased the disease specificity of the assay in UC patients from 70% to 92%, in accordance with recent reports in adults.[7,8,21] The combined use of both assay systems as an IBD panel gave an overall agreement of 68% with the final diagnosis (CD, UC, or non-IBD) on the basis of clinical, endoscopic, radiographic, and histopathologic criteria.

As in adults, the clinical presentation of IBD in the pediatric age group depends largely on the site and extent of the mucosal inflammation.[1] Insidious onset of abdominal pain, chronic diarrhea, and weight loss are hallmark symptoms of CD, whereas rectocolonic inflammation due to UC or CD usually presents more acutely with hematochezia. However, the diagnosis of IBD is often delayed in pediatric patients because of the nonspecific nature of symptoms.[1,6] These include decelerated growth velocity, anorexia with fatigue, or pubertal delay. Furthermore, extraintestinal manifestations such as arthritis or fever are more frequent in children than in adults, potentially misdirecting the initial clinical evaluation.[1,6] The results of this study suggest that this noninvasive IBD panel could be useful in suspected cases of CD and UC. In the subset of 36 patients prospectively evaluated for the possibility of IBD, 71% of patients with CD and 75% of those with UC had positive findings of a disease-specific panel at the time of their initial diagnostic workup. Although the CD-DX-1 and UC-DX-1 assays are imperfect, their sensitivities of the CD-DX-1 and UC-DX-1 assays are vastly superior to that reported for other commonly used biological disease markers, such as the rheumatoid factor (15%–20% sensitivity) or antinuclear antibody test (24%–66%) for juvenile rheumatoid arthritis.[22] About two thirds of pediatric patients with CD have an elevated erythrocyte sedimentation rate.[23] However, this widely used biological marker lacks disease specificity and is not useful in discriminating between CD and UC.

A major diagnostic dilemma in the pediatric age group results from the overlap between UC and CD limited to the large bowel. Most often, such cases are grouped as IC.[1,4] In the present study, we classified our patients into well-defined CD or UC groups. The IC classification was used when the endoscopic findings and histopathologic features of the biopsy specimens did not clearly distinguish between the other two entities. We observed that 13% of children with CD were positive for pANCA, consistent with the 10%–30% rate in patients with CD who express serum pANCA in adult IBD cohorts.[24,25] Looking at the CD-colitis subgroup, the percentage of pANCA-positive children (12%) was significantly inferior to that noted for the UC group (57%). Moreover, the UC-DX-1 assay proved to be highly specific (97%) for UC vs. CD and non-IBD colitis, with positive and negative predictive values of 91% and 79%, respectively (Table 4).

In our series, the majority (59%) of CD patients with pANCA immunofluorescence presented characteristic UC-like symptoms. Vasiliauskas et al.[3] recently noted that all pANCA-positive adult patients with CD had UC-like symptoms. The observation of pANCA in young children with CD at the onset of their disease supports the concept of a biological marker in a distinct subgroup of patients with CD. Furthermore, positive pANCA titers in the majority of pediatric patients with UC who had undergone surgery persisted, in agreement with findings of other recent studies.[26] The absence of pANCA in acute, self-limited, or eosinophilic colitis indicates that this disease marker does not simply reflect the presence of mucosal inflammation but more likely the type of mucosal inflammation. Similarly, ASCA titers were negative in all of our untreated celiac patients, in contrast to results of a previous report using a different method in adults.[27]

Among the group of children with IC, 22% had a positive UC-DX-1 panel vs. only 12% and 0% for the CD and non-IBD groups, respectively. These data suggest that children presenting with mild forms of UC are more likely to be classified initially as IC on clinical grounds. During follow-up, one of our IC pANCA-positive patients later proved to have UC.

The CD-DX-1 assay was able to clearly discriminate between CD colitis and IC, having 100% specificity for IgA and/or IgG ASCA positivity in CD patients with colitis. The only child with positive ASCA titers, initially classified as IC, was later confirmed to have CD. These data allow us to speculate that results of this assay, if positive, help in identifying and classifying patients with

CD who initially present with nonspecific colonic inflammation. Furthermore, the CD-DX-1 was 100% predictive value positive for CD colitis vs. UC and non-IBD colitis when the IgA and IgG ASCA were both positive.

In contrast to persistent pANCA results in patients with UC after colectomy, ASCA titers decreased toward normal values found in non-IBD and healthy controls when tested a few years after resection in pediatric patients with CD. This suggests the possibility that ASCA represents a humoral immune response to a luminal antigen, taken up across the inflamed, disrupted mucosal barrier. However, a prospective analysis of serum ASCA titers before and after surgery in a larger cohort of patients with CD is needed to confirm these findings. On the other hand, it is notable that patients with CD do not have higher antibody titers to *Candida* and other yeast organisms present within the gut lumen compared with healthy controls.[28] In view of negative ASCA titers in our untreated celiac patients, altered barrier function alone is an insufficient explanation. We did not observe a relationship between disease activity and ASCA positivity (Table 1). However, prospective testing serially over a defined period would have to be performed for firm conclusions on this issue to be drawn.

Our data on the combined use of the noninvasive CD-DX-1 and UC-DX-1 assays (Tables 2–4) suggest that they are helpful in the diagnosis of IBD in children. When the results of both tests are taken together, we observed a specificity of 95% and a positive predictive value of 96% for IBD. The diagnostic value of the combined use of these serological tests has been shown in a recent preliminary report in an adult cohort with IBD.[29] The high disease specificity of both diagnostic panels may be of particular assistance in making major therapeutic decisions, such as in patients with severe colitis. Positive CD-DX-1 and negative UC-DX-1 panel findings would favor the use of medical rather than surgical therapy in the nontoxic patient. Further prospective studies are needed to ascertain the predictive value and cost-effectiveness of using ASCA in combination with other laboratory markers in screening for IBD in patients with nonspecific symptoms and normal results of a physical examination.

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Comparative Study of ASCA (Anti–*Saccharomyces cerevisiae* Antibody) Assays in Inflammatory Bowel Disease

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Background & Aims: Anti–*Saccharomyces cerevisiae* antibody (ASCA) is a serologic marker associated with Crohn's disease (CD). Although there is still discussion on its clinical value, several companies each promote their own ASCA assay to be used in the gastroenterologist's practice at considerable expense. The aim of this study was to determine whether different ASCA assays agree sufficiently well for the results to be used interchangeably. **Methods:** Blood obtained from a large cohort of IBD patients with inflammatory bowel disease (IBD; 100 with CD, 100 with ulcerative colitis [UC]) and 178 controls (100 healthy blood donors and 78 patients with non-IBD diarrheal illnesses) was studied with 4 different ASCA assays. Sensitivity, specificity, and positive predictive value were compared. Agreement between assays was evaluated. **Results:** Sensitivity of ASCA for CD ranged between 41% and 76%. Sensitivity was inversely related to specificity and positive predictive value. Results correlated well overall (range = 0.54–0.90) and the different ROC curves showed good agreement. When recalculated cutoff points were used, interchangeability increased. However, large differences were seen when absolute values were compared. **Conclusions:** A large range in sensitivities and specificities of ASCA for CD is seen with different ASCA assays, mainly as a consequence of the cutoff value chosen for each individual assay. Although agreement between and within assays is good, caution is important when absolute values are used. Standardization of ASCA measurements is greatly needed.

There is great interest in serologic markers in inflammatory bowel disease (IBD). ASCAs (Anti–*Saccharomyces cerevisiae* antibodies) are antibodies with a high specificity for Crohn's disease (CD).[1–6] Their exact origin, as well as the epitope against which they are directed, is unclear. Increased prevalence in unaffected relatives of IBD patients and intrafamilial concordance favor a genetic origin of this antibody.[7,8] Why these antibodies occur in only a subset of patients with CD is unknown.

Although there is no consensus yet on the clinical value of serologic antibodies such as ASCA in IBD, several companies have developed an ASCA assay and have been promoting it as a noninvasive diagnostic tool in the gastroenterologist's practice. However, before a diagnostic test can be used in clinical practice, both a high sensitivity and specificity for the disease under investigation are needed. If the aim is screening subjects at risk for IBD, a high sensitivity is of great importance. If the test is used for differentiating between phenotypes, high specificity is necessary.

The reported sensitivity and specificity for ASCA in CD range between 55% and 65% and between 80% and 95%, respectively, but data are relatively scarce and originate from different centers all using different assays.[4,5] Therefore, before drawing general conclusions based on the results, we need to assess whether the different (commercial) assays for ASCA yield comparable results.

The main aim of this study was to compare the results of ASCA assays in a large cohort of IBD patients and controls. We wanted to assess agreement by comparing sensitivity, specificity, and positive predictive value for each individual assay and assess comparison of individual test results.

Materials and Methods

Study Population

Analyses were performed in a cohort of 200 unselected patients with IBD and 178 controls, using the different ASCA assays. The IBD patients included 100 patients with CD (57 female, 43 male; mean age, 38.5 years; age range, 15–78 years)

Abbreviations used in this paper: ASCA, anti–*Saccharomyces cerevisiae* antibody; BI, binding index; EU, ELISA unit; HRP, horseradish peroxidase; pANCA, perinuclear antineutrophil cytoplasmic antibody; ROC curve, receiver operating characteristic curve.

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Table 1. Clinical Characteristics of the Study Population

	CD (n = 100)	UC (n = 100)
Age at onset (yr)	26.01 ± 10.69	29.34 ± 12.27
Localization		
Small bowel	41	
Colon	16	
Small bowel and colon	43	
Anal involvement	35	
Pancolitis		28
Left-sided		61
Proctitis		11
Previous surgery	60	10
Disease activity		
Active/inactive	30/70	37/63

and 100 with ulcerative colitis (UC; 46 female, 54 male; mean age, 40.6 years; age range, 18–73 years). The diagnosis was made according to the Lennard-Jones criteria,[9] and all patients were carefully classified. Clinical characteristics are summarized in Table 1.

The control group consisted of 100 healthy individuals (54 female, 46 male; mean age, 38.0 years; age range, 21–74 years) with no family history of IBD and no immune-mediated disorders, all free from intestinal complaints, and 78 patients with non-IBD diarrheal illnesses (32 female, 46 male; mean age, 39.5 years; age range, 18–94 years). This group consisted of patients with diverticulitis (n = 33), infectious gastroenteritis (n = 32), ischemic colitis (n = 4), acute self-limiting colitis (n = 3), pseudomembranous colitis (n = 2), and other aspecific colitis (n = 4). Patients and controls were matched for age and sex. All subjects originated from and were living in the northern part of Belgium. Whole venous blood was obtained, and serum was separated after clotting by centrifugation. Aliquots were then taken and were stored at −30°C until the tests were performed.[10] The investigators were blinded to disease status at the time of the ASCA assays. Approval for the study was given by the ethical committee of the University of Leuven (Belgium).

ASCA ELISA Assays

Four ASCA assays were compared: the assay developed by Dr. D. Poulain (Laboratoire de Parasitologie-Mycologie, Centre Hospitalier Regional Universitaire, Lille, France) and 3 commercially available assays from Prometheus Laboratories Inc. (San Diego, CA); Medipan Diagnostica (Selchow, Germany), distributed by Euribel S.A./N.V. (Brussels, Belgium); and Quanta Lite ASCA (Inova Diagnostics, San Diego, CA), distributed by Medigal S.A. (Villers-Poterie, Belgium).

ASCA ELISA assay—Dr. D. Poulain, CHRU, Lille, France. This standardized ELISA uses the crude mannan from *Saccharomyces cerevisiae uvarum I* as the antigen. Plates were coated with phosphopeptidomannan extracted from yeast cells from cultures in bioreactors. One hundred microliters of serum diluted 1:1000 in Tris-natrium-Tween was added and incubated at 37°C for 1 hour. On each plate, 1 serum that was strongly positive and 1 completely negative against the *S. cerevisiae* mannan were added. After 5 washing steps, 100 μL of alkaline phosphatase–labeled goat anti-human immunoglobulin (Ig) G, IgA, IgM (heavy and light chains) diluted 1:1000 was added (Zymed Laboratories, Inc., San Francisco, CA), followed by incubation at 37°C for another hour and the same extensive washing procedure. To obtain a color reaction, 100 μL of Magia substrate (Merck, Belgolabo, Belgium) for alkaline phosphatase was added, and plates were put in the dark for 30 minutes. Reading was then done at 405 nm on an Anthos ht II (VEL, Leuven, Belgium) automatic photometer. Absorbance of the individual sera was expressed relative to the absorbance of a pool of sera collected from well-characterized patients with CD. Based on standard reactivity curves (ROC) from Dr. Poulain, samples with ELISA values of ≥3.12 were considered positive.

ASCA ELISA assay—Prometheus Laboratories Inc., San Diego, CA. For this assay, the uncoded samples were sent to Prometheus Laboratories Inc., where the assay was performed. Prometheus Laboratories advocates a combination of ASCA and UC-specific perinuclear antineutrophil cytoplasmic antibody (pANCA) tests as a means of improving the accuracy of the diagnosis of IBD and to better discriminate between CD and UC, as well as to stratify the potential disease process in positive patients. Microtiter plates coated with phosphopeptidomannan from the yeast *S. cerevisiae* were used. Controls and samples were added at 1:100 dilution. After the unbound serum was washed off, bound antibodies were labeled with alkaline phosphatase–conjugated goat anti-human IgG or IgA. After the addition of *p*-nitrophenol, specific absorbance was measured at 405 nm. The absorbance of each sample was evaluated and assigned ELISA unit (EU) values relative to the absorbance of a pool of sera collected from well-characterized patients with CD. The standard pool was arbitrarily assigned the value of 100 EU/mL. Results were positive if results of the anti-IgG, the anti-IgA assay, or both were positive. The cutoff for positivity as determined by the company on the basis of the results in well-defined patients with CD was set at 20 EU/mL and 40 EU/mL for IgA and IgG ASCA, respectively.

ASCA ELISA assay—Medipan Diagnostica, Selchow, Germany. Diluted patient and control samples reacted with mannan immobilized on the solid phase of a microtiter plate. Serum was diluted 1:50 and added to the microtiter plates. After an incubation period of 60 minutes at 37°C, unbound serum components were removed by a washing step. The antibodies bound then specifically reacted with anti-human IgG or IgA antibodies conjugated to horseradish peroxidase (HRP). An incubation period of 30 minutes at 37°C was followed by a washing step. The enzyme reaction was stopped by dispensing of an acidic solution (H_2SO_4) into the wells after 10 minutes at room temperature, turning the solution from blue to yellow. Plates were read at 450 nm. To each microtiter plate, 1 ASCA-positive and 1 ASCA-negative control and 1 cutoff control (20 U/mL) were added, calculated from ROC curves. Qualitative evaluation of the results was assessed by calculating the binding index (BI): BI = OD

(sample)/OD (cutoff control). ASCA IgG and IgA were considered positive at BI ≥1.0.

QUANTA Lite ASCA IgG and IgA ELISA assay—Inova Diagnostics, San Diego, CA. Partially purified and disrupted *S. cerevisiae* was bound to the wells of a polystyrene microtiter plate. One hundred microliters of serum at a dilution of 1:101 (HRP diluent containing Tris-buffered saline, Tween 20, absorbants, and protein stabilizers) was added to the wells. After incubation for 30 minutes at room temperature, unbound sample was washed away and 100 μL of HRP IgG or IgA conjugate (goat anti-human) was added. After another 30 minutes' incubation at room temperature, 100 μL of TMB Chromogen was added to each well, and the plate was incubated again (in the dark) for 30 minutes at room temperature. Absorbance (OD) was read at 450 nm after addition of 100 μL of stop solution (HRP stop solution, 0.344 mol/L sulfuric acid). On each plate, 1 high positive, 1 low positive, and 1 negative control were added. To interpret results correctly, the OD of the high positive must be >1, and that of the ASCA-negative control cannot be >0.2. The ASCA low positive has to be more than 2× the negative control, or >0.2. Reactivity was determined by the following formula: Sample OD/ELISA Low Positive OD × 25 (25 is the number of units assigned to the ASCA IgG ELISA low positive). Results were expressed as negative (0–20.0 U), equivocal (20.1–24.9 U), or positive (≥25 U). Equivocal specimens were retested until an unequivalent result was obtained.

Statistical Analysis

Sensitivity for each test result was defined as the probability of a positive test result in a patient with the disease under investigation. Specificity was defined as the probability of a negative test result in a patient without the disease under investigation. The positive predictive value was defined as the probability of being affected with the disease in a patient with a positive test result. Receiver operating characteristic curves (ROC) were generated by plotting sensitivity (y-axis) vs. 1 −specificity (x-axis) or true-positive rates vs. false-positive rates.[11,12] By this technique, one can pinpoint the decision level at which optimal sensitivity and specificity can be achieved. A "hidden" third axis is contained in the curve itself: the curve is drawn through points that represent different decision cutoff levels. The whole curve is a graphic display of the performance of a test. The area under the curve describes the test's overall performance; hence, when the ROC curves of several tests are superimposed, the most predictive test can be selected. The strength of the ROC graphic lies in its providing a meaningful comparison of different tests. When the initial publication of an assay presents a cutoff for analysis purposes, the assay is often categorized as sensitive or specific based on this cutoff. Every assay can be as sensitive as desired at some cutoff and as specific as desired at another cutoff.

Linear correlation between the different assays was performed using Pearson's correlation. Correlation was measured under the null hypothesis of no relationship. However, correlation between the results of 2 measurements as an indicator of agreement can be misleading because a high correlation does not necessarily mean that the 2 methods agree. A change of scale in measurements does not affect correlation but affects agreement, so the 2 methods cannot be mixed. Correlation also depends on the range of the true quantity in the sample, and hence a large range of values shows a higher correlation than a low range. To avoid this misinterpretation, we performed Altman–Bland analysis,[13,14] which is done by plotting the differences between observations against the means. Good agreement should show a horizontal curve, indicating a stable difference between observations for increasing means.

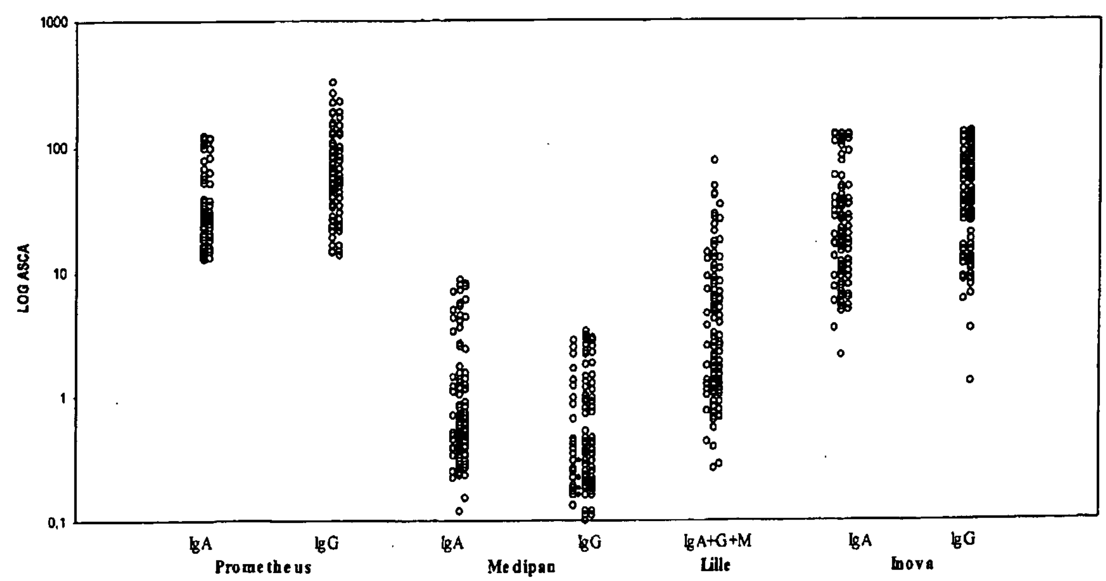

Figure 1. Range of ASCA values in the different assays (patients with CD).

Table 2. Number of ASCA IgA-Positive Samples and IgG-Positive Samples in Patients With IBD and Controls for the Different Assays (% or Absolute Numbers for All, Except Inflammatory Controls)

	CD (n = 100)			UC (n = 100)			Controls (n = 178): Healthy (n = 100)			Controls (n = 178): Inflammatory (n = 78)		
	IgA	IgG	IgA or IgG	IgA	IgG	IgA or IgG	IgA	IgG	IgA or IgG	IgA	IgG	IgA or IgG
Prometheus	50	52	61	7	2	7	2	3	4	5 (6.4%)	2 (2.6%)	6 (7.7%)
Medipan	34	26	41	2	0	2	1	0	1	3 (3.8%)	1 (1.3%)	4 (5.1%)
Lille			45			5			3			8 (10.3%)
Inova	56	75	76	3	13	14	1	5	6	4 (5.1%)	8 (10.3%)	12 (15.4%)

The reproducibility of each individual kit was assessed by testing all serum samples at least twice. Pearson and Altman–Bland analysis were then carried out to determine correlation between different measurements. Multivariate analysis (SAS/STAT release 6.11 edition 1; SAS Institute Inc., Raleigh, NC) was performed to ascertain if specific clinical characteristics were associated with ASCA.

Results

The range of ASCA titers and mean ASCA in CD varied substantially between the different assays. For the Prometheus Laboratories assay, mean ASCA IgA was 51.80 (±38.9; range, <12.5–121.3) and mean ASCA IgG was 80.6 (±70.0; range, <12.5–323.2). Using the Medipan assay, mean ASCA IgA was 1.51 (±2.0; range, 0.12–8.44) and ASCA IgG was 0.81 (±0.85; range, 0.08–3.3). The ASCA results from Lille ranged from 0.26 to 76.29, with a mean titer of 7.23 (±11.5). Inova mean ASCA IgA was 42.34 (±41.35; range, 2.12–124.4) and IgG was 52.05 (±37.4; range, 1.29–131.73) (Figure 1).

Excellent reproducibility of measurements was achieved for all 4 assays (mean Pearson $r = 0.998$; range, 0.98–1; mean Altman–Bland $r = 0.18$; range, 0.09–0.27). The prevalence of ASCA in CD ranged from 41% to 76% (Table 2 and Figure 2). Accordingly, sensitivity, specificity and positive predictive value for each assay showed variation between assays (Table 3).

The highest sensitivity (76%) was seen with the Inova assay, but this assay also had the lowest specificity. Lower sensitivity was seen with both the Medipan (41%) and Lille (45%) assays than with the Inova, but specificity for these assays was the highest. The sensitivity (61%) and specificity (93%–94%) of the Prometheus Laboratories assay were situated between those of the other assays.

Of all ASCA-positive UC patients (IgA or IgG), 1 patient had positive results in all 4 assays, 2 patients had positive results in 3 assays, 3 patients had positive results in 2 different assays, and 12 patients had positive results in 1 assay. There were UC patients who had positive ASCA IgA results in all studied assays. No clinical similarities in age at onset, localization of disease, clinical course, therapy, or surgery were seen in these ASCA-positive UC patients. Among the healthy controls, 1 subject had positive results in 3 assays, 2 in 2 assays, and 7 in 1 assay. For the inflammatory controls, 3 subjects had positive ASCA results in 3 assays, 6 in 2 assays, and 9 in 1 assay. ASCA was not related to a specific diarrheal illness. Using multivariate analysis, no association was seen between ASCA and clinical characteristics such as

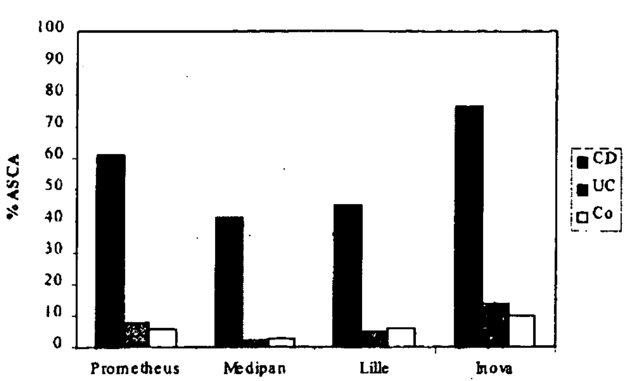

Figure 2. Prevalence of ASCA in patients with CD and UC and controls in the different assays.

Table 3. Sensitivity, Specificity, and Positive and Negative Predictive Value of ASCA for Differentiating CD From Non-CD and CD From UC

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CD vs. non-CD				
Prometheus	61.0	93.9	78.2	87.0
Medipan	41.0	97.5	85.4	82.1
Lille	45.0	94.4	73.8	82.6
Inova	76.0	88.5	70.4	91.1
CD vs. UC				
Prometheus	61.0	93.0	89.7	70.5
Medipan	41.0	98.0	95.3	62.4
Lille	45.0	95.0	90.0	63.3
Inova	76.0	86.0	84.4	78.2

PPV, positive predictive value; NPV, negative predictive value.

Table 4. Pearson Correlation Coefficients of ASCA Between and Within Assays

		Prometheus		Medipan		Lille	Inova	
		IgA	IgG	IgA	IgG	IgA + G + M	IgA	IgG
Prometheus	IgA	1						
	IgG	0.67	1					
Medipan	IgA	0.85	—	1				
	IgG	—	0.71	0.71	1			
Lille	IgA+G+M	0.59	0.75	0.65	0.76	1		
Inova	IgA	0.90	—	0.82	—	0.54	1	
	IgG	—	0.78	—	0.74	0.66	0.61	1

CD activity, localization of disease, age at onset, or need for surgery.

When ASCA IgA and IgG levels in the different assays were compared, a good overall correlation was seen, ranging from 0.54 to 0.90, and a close relationship was also found between IgG and IgA titers within the different kits (Table 4). However, Altman–Bland analysis showed overall low agreement among assays, indicating that the differences between assays increased for increasing means (Figure 3).

Off all curves, the results of Inova corresponded most with those of Prometheus but still showed heteroscedasticity (increasing variation with increasing means). Heteroscedasticity was also seen when comparing the titers from Lille were compared with those from Inova and those from Prometheus, but not with those from Medipan.

ROC curves constructed for each of the 4 assays based on the present results showed very similar curves when they were plotted against each other (Figure 4). Based on the new calculated ROC curves, optimal cutoff values for ASCA IgA and IgG were calculated, searching for an optimal ratio for sensitivity and specificity. For Prometheus, this new cutoff value was 13.5 (IgG; cutoff proposed by the company, 40 EU/mL), for Medipan it was 0.39 (IgA) and 0.21 (IgG; proposed cutoff, BI ≥1), for Lille it was 1.23 (proposed cutoff, 3.12), and for Inova it was 10 (IgA) and 14.81 (IgG; proposed cutoff, >25 U). Calculated sensitivity and specificity for ASCA IgA based on these new cutoffs are 79% and 77% (Medipan), 74% and 74.1% (Lille), and 76% and 76.8% (Inova), respectively. For ASCA IgG based on these new cutoffs, sensitivity and specificity would be 79% and 82% (Prometheus), 76% and 77% (Medipan), 74% and

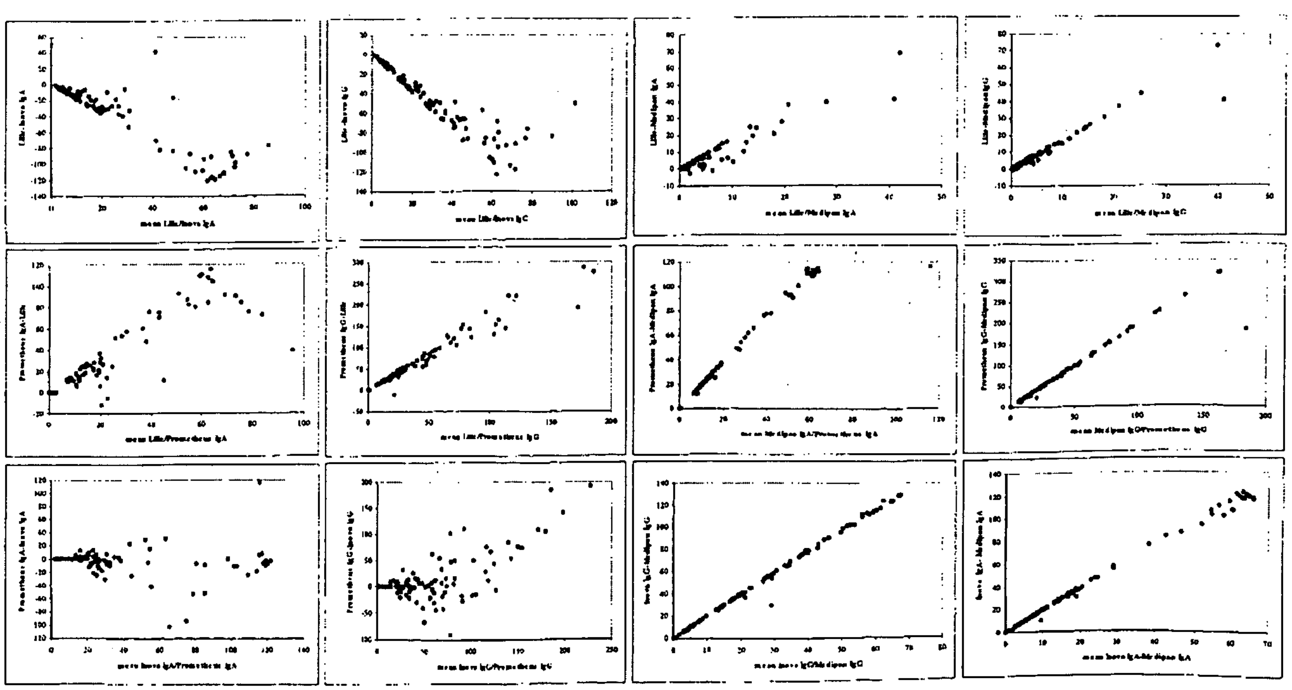

Figure 3. Altman–Bland analysis between the different assays.

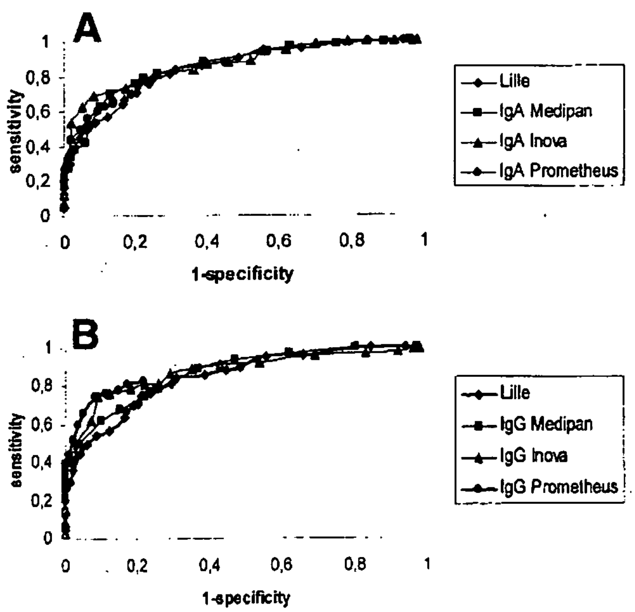

Figure 4. ROC curves (CD vs. non-CD) for each ASCA assay. (*A*) IgA; (*B*) IgG.

74.1% (Lille), and 79.8% and 79.8% (Inova), respectively.

Discussion

Antibodies against the yeast *S. cerevisiae*, a yeast commonly used in the food industry, are found in CD with a prevalence varying from 55% to 65%. The specificity for CD is higher, with reported figures of 80%–95%. However, data are still scarce and come from different research groups mostly using different assays. Nevertheless, several commercial companies recently developed an ASCA assay and promote its use in IBD clinical practice. The use of these tests involves an important financial investment; therefore, critical assessment is necessary.

In this study we investigated whether different ASCA assays agree well enough to be used interchangeably. Therefore, we compared 4 ASCA assays in a large population of IBD patients and controls.

The strength of this study lies in the fact that the same patients and controls were tested for all 4 assays and that serum from each subject originated from the same blood sampling. A large variation in sensitivity between the assays was seen, ranging from 41% to 76%. Specificity was inversely related to sensitivity.

One study showed that ASCA IgA was 100% specific for CD.[15] In the 27 patients with UC tested by these investigators, no positive ASCA IgA results were found. However, we could not replicate this in our larger study because there were patients with UC with positive results for ASCA IgA in all assays.

There are several possible explanations for the varying results among the studied kits. Because all serum from each patient came from the same blood sampling, differences caused by antibody fluctuations over time are excluded. Further, interoperator variance also does not account for the differences because the assays were all performed by the same persons with extensive experience in ELISA. Only the ASCA assay from Prometheus Laboratories was performed at the company itself, but the same assumptions can be made. A more plausible explanation for the varying results may lie in differences in the *S. cerevisiae* strains used, in the purification process, and in the way of coating the plates. Because the exact epitope structure is not yet known, actual coating is now done with partially purified and disrupted *S. cerevisiae* (Inova), oligopeptidomannans from the cell wall of the yeast (Lille and Prometheus), or the mannoses (Medipan). The dilution of the serum to be used, the conjugate, and the incubation time are also different in the 4 assays according to the manufacturer's conditions, which can also influence results. Maybe the parameter influencing sensitivity and specificity most is the determination of the cutoff value based on ROC curves. Although we showed that ROC curves overlapped remarkably well, indicating that the overall performance of the assays is very similar and that companies validated the cutoff value of their assays in similar ways, each company still chose a particular cutoff value, based on whether a more specific (higher cutoff) or more sensitive (lower cutoff) assay was looked for. Because differences in cutoff values can greatly influence the interpretation of a test result, gastroenterologists should be aware of the individual aims of the companies selling the assays. They should take both the sensitivity and specificity of each assay into account. With the 4 assays currently tested in this study, this issue becomes very clear. An assay with low sensitivity but high specificity will be useful for differentiating CD from UC. For population screening, a high sensitivity is pursued, and specificity is less critical. Based on the current data, we believe ASCA is not suited for screening purposes because of its low sensitivity. However, its high specificity and positive predictive value make ASCA a valuable marker for differentiating CD from UC. The current data did not support any relationship of ASCA with clinical characteristics such as age at onset, localization of disease, need for surgery, or disease activity, but the present cohort of CD patients may have been too small to detect such a relationship. Nevertheless, further analysis is needed to assess which

subgroup of CD patients express ASCA. This might then lead to increased clinical utility. Because the combination of ASCA and pANCA is advocated by different companies, the same comparative study should be carried out for pANCA assays.

From the Altman–Bland analysis it is clear that absolute ODs and ASCA titers should not be used interchangeably, and we propose that standardization of the cutoff interpretation is necessary.

In conclusion, the current available ASCA assays agree well but differ greatly in the interpretation of results. Therefore, caution should be used when comparing results. It is clear that cutoff points have been chosen for different purposes. Absolute values and absorbancies cannot be used interchangeably between assays before standardization.

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(54) **DIAGNOSIS, PREVENTION AND TREATMENT OF CROHN'S DISEASE USING THE OMPC ANTIGEN**

DIAGNOSE, PROPHYLAXE UND BEHANDLUNG VON MORBUS CROHN DURCH DIE VERWENDUNG DES OMPC ANTIGENS

DIAGNOSTIC, PREVENTION ET TRAITEMENT DE LA MALADIE DE CROHN AU MOYEN D'UN ANTIGENE OMPC

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Description

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0001] The invention relates generally to the fields of immunology and inflammatory bowel disease and more specifically to the diagnosis and treatment of Crohn's disease using the bacterial antigen, outer membrane protein C (OmpC).

BACKGROUND INFORMATION

[0002] Inflammatory bowel disease (IBD) is the collective term used to describe two gastrointestinal disorders of unknown etiology: Crohn's disease (CD) and ulcerative colitis (UC). The course and prognosis of IBD, which occurs world-wide and is reported to afflict as many as two million people, varies widely. Onset of IBD is predominantly in young adulthood with diarrhea, abdominal pain, and fever the three most common presenting symptoms. The diarrhea may range from mild to severe, and anemia and weight loss are additional common signs of IBD. Ten percent to fifteen percent of all patients with IBD will require surgery over a ten year period. In addition, patients with IBD are at increased risk for the development of intestinal cancer. Reports of an increased occurrence of psychological problems, including anxiety and depression, are perhaps not surprising symptoms of what is often a debilitating disease that strikes people in the prime of life.

[0003] Unfortunately, the available therapies for inflammatory bowel disease are few, and both diagnosis and treatment have been hampered by a lack of knowledge regarding the etiology of the disease. What is clear, however, is that a combination of genetic factors, exogenous triggers and endogenous microflora can contribute to the immune-mediated damage to the intestinal mucosa seen in inflammatory bowel disease. In Crohn's disease, bacteria have been implicated in initiation and progression of the disease: the intestinal inflammation in Crohn's disease is notable for its frequent responsiveness to antibiotics and susceptibility to bacterial fecal flow. Common intestinal colonists and novel pathogens have been implicated in Crohn's by direct detection or by disease associated anti-microbial immune responses. Furthermore, in many genetically susceptible animal models of chronic colitis, lumenal micro-organisms are a necessary cofactor for disease; animals housed in a germ-free environment do not develop colitis. However, despite much direct and indirect evidence for a role for enteric microorganisms in Crohn's disease, the pathogenic organisms and antigens contributing to the immune dysregulation seen in this disease have not been identified.

[0004] Current diagnostic assays for Crohn's disease are unable to detect all patients with the disease.
Thus, identification of novel microbial antigens associated with Crohn's disease would provide reagents that can increase the sensitivity of current diagnostic assays. In addition, such microbial antigens can bear a disease related T-cell epitope and, as original or contributing inducers of the disease-related immune response, can be effective tolerogenic antigens for treating Crohn's disease.

[0005] Thus, there is a need for identification of microbial antigens associated with Crohn's disease that can be used to improve the sensitivity of current diagnostic assays for this disease. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

[0006] The present invention provides a method of diagnosing Crohn's disease in a subject by determining the presence or absence of IgA anti-outer membrane protein C (OmpC) antibodies in a sample from the subject, where the presence of the IgA anti-OmpC antibodies indicates that the subject has Crohn's disease. A method of the invention can be practiced, for example, by contacting a sample from a subject with an OmpC antigen, or reactive fragment thereof, under conditions suitable to form a complex of the OmpC antigen, or reactive fragment thereof, and IgA antibody to the OmpC antigen; contacting the complex with an anti-IgA antibody; and detecting the presence or absence of IgA anti-OmpC antibodies, where the presence of the IgA anti-OmpC antibodies in the subject indicates that the subject has Crohn's disease. In one embodiment, the invention is practiced using an OmpC antigen having substantially the amino acid sequence of SEQ ID NO: 1. In another embodiment, the IgA anti-OmpC antibodies are detected with an enzyme-linked immunosorbent assay.

[0007] The invention further provides a method of diagnosing Crohn's disease by determining the presence or absence of IgA anti-OmpC antibodies in a sample from the subject and the presence or absence of IgA anti-*Saccharomyces cerevisiae* antibodies (ASCA) in a sample from the subject, where the presence of IgA anti-OmpC antibodies or the presence of IgA ASCA in the sample from the subject each independently indicates that the subject has Crohn's disease. The presence of IgA ASCA can be determined, for example, by reactivity with purified yeast cell wall phos-

phopeptidomannan (PPM), which can be prepared, for example, from ATCC strain #38926.

[0008] The invention additionally provides a method of diagnosing Crohn's disease by determining the presence or absence of IgA anti-OmpC antibodies in a sample from the subject and the presence or absence of IgA anti-I-2 polypeptide antibodies in a sample from the subject, where the presence of IgA anti-OmpC antibodies or the presence of IgA anti-I-2 polypeptide antibodies in the sample from the subject each independently indicates that the subject has Crohn's disease. In one embodiment, the presence of IgA anti-I-2 polypeptide antibodies is determined by IgA reactivity against an I-2 polypeptide having substantially the amino acid sequence of SEQ ID NO: 3.

[0009] Further provided by the invention is a method of diagnosing Crohn's disease in a subject by determining the presence or absence of IgA anti-OmpC antibodies in a sample from the subject; determining the presence or absence of IgA ASCA in a sample from the subject; and determining the presence or absence of IgA anti-I-2 polypeptide antibodies in a sample from the subject, where the presence of the IgA anti-OmpC antibodies, the presence of IgA ASCA or the presence of IgA anti-I-2 polypeptide antibodies each independently indicates that the subject has Crohn's disease. In one embodiment, this method further includes the step of determining the presence or absence of perinuclear anti-neutrophil antibodies (pANCA) in a sample from the subject.

[0010] The invention further provides the use of an OmpC antigen, or tolerogenic fragment thereof, for preparing a medicament to induce tolerance in a patient with Crohn's disease. An OmpC antigen useful for a medicament for inducing tolerance can have, for example, substantially the amino acid sequence of SEQ ID NO: 1.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011]

Figure 1 shows the IgA OmpC reactivity and IgA I-2 reactivity of sera from patients having Crohn's disease.

Figure 2 shows IgA OmpC reactivity and IgA ASCA reactivity of sera from patients having Crohn's disease.

Figure 3 shows IgA OmpC reactivity and IgG OmpC reactivity of sera from patients having Crohn's disease.

Figure 4 shows IgA and IgG OmpC reactivity of sera from normal individuals.

Figure 5 shows the *E. coli* OmpC amino acid sequence (SEQ ID NO: 1).

Figure 6 shows the I-2 nucleotide sequence (SEQ ID NO: 2) and predicted amino acid sequence (SEQ ID NO: 3).

DETAILED DESCRIPTION OF THE INVENTION

[0012] The present invention is directed to the exciting discovery that IgA antibodies to outer membrane protein C (OmpC) are associated with Crohn's disease. As shown in Figure 4, only a single individual without Crohn's disease ("normal") had IgA OmpC reactivity greater than 0.2_{405}, although many normals showed significant IgG OmpC reactivity. In contrast to the normals, significant IgA OmpC reactivity was present in sera from many patients having Crohn's disease (see Figure 1). Furthermore, this reactivity was apparently distinct, in large part, from IgA I-2 and IgA ASCA reactivity seen in sera from Crohn's disease patients (Figures 1 and 2). These results are summarized in Table 1, which indicates that IgA OmpC reactivity together with IgA ASCA reactivity, pANCA reactivity and I-2 polypeptide reactivity is a highly sensitive diagnostic system which can detect 86% of patients with Crohn's disease. Furthermore, as shown in Table 2, IgA OmpC reactivity itself detected 55% of patients having Crohn's disease. These results indicate that IgA OmpC reactivity can be valuable in increasing the number of Crohn's disease patients that are diagnosed with the disease, thereby facilitating earlier and more appropriate treatment.

TABLE 1

CD COHORT							
	Total	ASCA Panel+	IgA OmpC+	pANCA	I2	Additional % Detected	Cumulative % Detected
CD Cohort	153	86				56%	56%
ASCA Panel -	67		31			20%	75%
ASCA Panel -/ OmpC-	36			12		8%	84%

TABLE 1 (continued)

CD COHORT							
	Total	ASCA Panel+	IgA OmpC+	pANCA	I2	Additional % Detected	Cumulative % Detected
ASCA Panel -/ OmpC-/ pANCA-	24				2	1%	86%
Total % Detected:						86%	86%

TABLE 2

CD COHORT					
	Total	ASCA Panel+	OmpC+	pANCA	I2
n	153	86	84	36	79
% detected:		56%	55%	24%	52%

[0013] Based on the above, the present invention provides a method of diagnosing Crohn's disease in a subject by determining the presence or absence of IgA anti-OmpC antibodies in a sample from the subject, where the presence of the IgA anti-OmpC antibodies indicates that the subject has Crohn's disease. A method of the invention can be practiced, for example, by contacting a sample from a subject with an OmpC antigen, or reactive fragment thereof, under conditions suitable to form a complex of the OmpC antigen, or reactive fragment thereof, and IgA antibody to the OmpC antigen; contacting the complex with an anti-IgA antibody; and detecting the presence or absence of IgA anti-OmpC antibodies, where the presence of the IgA anti-OmpC antibodies in the subject indicates that the subject has Crohn's disease. In one embodiment, the invention is practiced using an OmpC antigen having substantially the amino acid sequence of SEQ ID NO: 1. In another embodiment, the IgA anti-OmpC antibodies are detected with an enzyme-linked immunosorbent assay.

[0014] The outer-membrane protein C ("OmpC") useful in the methods of the invention is a "porin," a class of transmembrane proteins that are found in the outer membranes of bacteria, including gram-negative enteric bacteria such as *E. coli*. The porins in the outer membrane of an *E. coli* cell provide channels for passage of disaccharides, phosphate and similar molecules. Porins can be trimers of identical subunits arranged to form a barrel-shaped structure with a pore at the center (Lodish et al., Molecular Cell Biology, Chapter 14 (1995)).

[0015] OmpC is one of the major porin proteins found in the outer membranes of bacteria such as *E. coli*. OmpC is similar in structure and function to outer-membrane protein F ("OmpF"). Both assemble as trimers in the outer membrane to form aqueous channels that allow the passive diffusion of small, hydrophilic molecules across the hydrophobic barrier. However, OmpC pores have a diameter of 1.1 nm, while OmpF pores have a diameter of 1.2 nm. This difference results in a slower rate of diffusion through the OmpC pores than through the OmpF pores.

[0016] Porin expression can be influenced by environmental conditions, including osmolarity, temperature, growth phase and toxin concentration. For example, in the intestine, where both nutrient and toxic molecule concentrations are relatively high, OmpC, with a smaller pore diameter, is the predominant porin (Pratt et al., Mol. Micro., 20:911-917 (1996)).

[0017] As used herein, the term "OmpC antigen" or "OmpC" means a protein that has linear or conformational homology to OmpC. A OmpC antigen can be derived from a gram-negative bacterium, such as *E. coli*, and can be a species homolog of *E. coli* OmpC (SEQ ID NO: 1), which is shown in Figure 5. A similar sequence for an OmpC antigen has been published by Miranno et al. (JBC, 258:6932-6940 (1983)). In nature, an OmpC antigen is a protein that forms a trimeric structure in the outer membrane of bacteria which allows the passage of small molecules, or a precursor of such a protein.

[0018] As used herein, the term OmpC antigen or OmpC means a protein that has at least 50% amino acid identity with *E. coli* OmpC (SEQ ID NO: 1) shown in Figure 5. An OmpC antigen can have, for example, at least 60%, 70%, 80%, 85%, 90% or 95% amino acid identity with SEQ ID NO: 1, said amino acid identity determined with CLUSTALW using the BLOSUM 62 matrix with default parameters. For use in the methods of the invention, an OmpC antigen can be partially purified, for example, by spheroplast lysis from OmpA and OmpF deficient cells as described in Example IV, or can similarly prepared from a variety of *E. coli* strains, which can contain OmpA and OmpF in addition to OmpC. An OmpC antigen also can be prepared recombinantly by expressing an encoding nucleic acid sequence such as that available as GenBank accession K00541 by methods well known in the art (see, for example, Ausubel et al., Current

Protocols in Molecular Biology John Wiley & Sons, Inc. New York (1999)).

[0019] The methods of the invention relate to determining the presence or absence of IgA anti-OmpC antibodies in a subject. As used herein, the "presence of IgA anti-OmpC antibodies" means IgA reactivity against an OmpC antigen that is greater than two standard deviations above the mean IgA anti-OmpC reactivity of control (normal) sera analyzed under the same conditions.

[0020] The methods of the invention relate to the diagnosis and treatment of Crohn's disease (regional enteritis), which is a disease of chronic inflammation that can involve any part of the gastrointestinal tract. Commonly the distal portion of the small intestine (ileum) and cecum are affected. In other cases, the disease is confined to the small intestine, colon or anorectal region. Crohn's disease occasionally involves the duodenum and stomach, and more rarely the esophagus and oral cavity.

[0021] The variable clinical manifestations of Crohn's disease are, in part, a result of the varying anatomic localization of the disease. The most frequent symptoms of Crohn's disease are abdominal pain, diarrhea and recurrent fever. Crohn's disease is commonly associated with intestinal obstruction or fistula, which is an abnormal passage between diseased loops of bowel, for example. Crohn's disease also includes complications such as inflammation of the eye, joints and skin; liver disease; kidney stones or amyloidosis. In addition, CD is associated with an increased risk of intestinal cancer.

[0022] Several features are characteristic of the pathology of Crohn's disease. The inflammation associated with CD, known as transmural inflammation, involves all layers of the bowel wall. Thickening and edema, for example, typically also appear throughout the bowel wall, with fibrosis also present in long-standing disease. The inflammation characteristic of CD also is discontinuous in that segments of inflamed tissue, known as "skip lesions," are separated by apparently normal intestine. Furthermore, linear ulcerations, edema, and inflammation of the intervening tissue lead to a "cobblestone" appearance of the intestinal mucosa, which is distinctive of CD.

[0023] A hallmark of Crohn's disease is the presence of discrete aggregations of inflammatory cells, known as granulomas, which are generally found in the submucosa. Some Crohn's disease cases display the typical discrete granulomas, while others show a diffuse granulomatous reaction or nonspecific transmural inflammation. As a result, the presence of discrete granulomas is indicative of CD, although the absence of granulomas also is consistent with the disease. Thus, transmural or discontinuous inflammation, rather than the presence of granulomas, is a preferred diagnostic indicator of Crohn's disease (Rubin and Farber, Pathology (Second Edition) Philadelphia: J.B. Lippincott Company (1994)).

[0024] In contrast to ulcerative colitis, which is characterized by a continuous inflammation of the colon that usually is more severe distally than proximally, Crohn's disease is a patchy disease with frequent sparing of the rectum. Furthermore, the inflammation is Crohn;s disease is distinct from the superficial inflammation seen in ulcerative colitis, which is usually limited to the mucosal layer and is characterized by an acute inflammatory infiltrate with neutrophils and crypt abscesses. Instead, Crohn's disease affects the entire thickness of the bowel wall with granulomas often, although not always, present. Furthermore, involvement of the terminal ileum, a cobblestone-like appearance, discrete ulcers or fistulas suggest Crohn's disease. Characteristics that serve to distinguish Crohn's disease from ulcerative colitis are summarized in Table 3 (Rubin and Farber, *supra,* 1994).

Table 3

Characteristic Features of Crohn's disease and ulcerative colitis		
Feature	**Crohn's Disease**	**Ulcerative Colitis**
Macroscopic		
Thickened bowel wall	Typical	Uncommon
Luminal narrowing	Typical	Uncommon
"Skip" lesions	Common	Absent
Right colon predominance	Typical	Absent
Fissures and fistulas	Common	Absent
Circumscribed ulcers	Common	Absent
Confluent linear ulcers	Common	Absent
Pseudopolyps	Absent	Common
Microscopic		
Transmural inflammation	Typical	Uncommon

Table 3 (continued)

Characteristic Features of Crohn's disease and ulcerative colitis		
Feature	**Crohn's Disease**	**Ulcerative Colitis**
Microscopic		
Submucosal fibrosis	Typical	Absent
Fissures	Typical	Rare
Granulomas	Common	Absent
Crypt abscesses	Uncommon	Typical

[0025] As used herein, the term "subject" means any animal capable of having inflammatory bowel disease, including a human, non-human primate, rabbit, rat or mouse, especially a human. A subject can have one or more symptoms of Crohn's disease or ulcerative colitis, or may be asymptomatic.

[0026] A sample useful in the methods of the invention can be obtained from any biological fluid having antibodies such as, for example, whole blood, plasma, saliva, or other bodily fluid or tissue, preferably serum.

[0027] As used herein, the term "fragment" means a peptide, polypeptide or compound containing naturally occurring amino acids, non-naturally occurring amino acids or chemically modified amino acids. A reactive fragment or tolerogenic fragment of an OmpC antigen also can be a peptide mimetic, which is a non-amino acid chemical structure that mimics the structure of a peptide having an amino acid sequence, provided that the peptidomimetic retains preferential reactivity with IgA antibodies in sera of Crohn's disease patients or tolerogenic activity, as defined herein. Such a mimetic generally is characterized as exhibiting similar physical characteristics such as size, charge or hydrophobicity in the same spatial arrangement found in its peptide counterpart. A specific example of a peptide mimetic is a compound in which the amide bond between one or more of the amino acids is replaced, for example, by a carbon-carbon bond or other bond well known in the art (see, for example, Sawyer, Peptide Based Drug Design, ACS, Washington (1995)).

[0028] As used herein, the term "amino acid" refers to one of the twenty naturally occurring amino acids, including, unless stated otherwise, L-amino acids and D-amino acids. The term amino acid also refers to compounds such as chemically modified amino acids including amino acid analogs, naturally occurring amino acids that are not usually incorporated into proteins such as norleucine, and chemically synthesized compounds having properties known in the art to be characteristic of an amino acid, provided that the compound can be substituted within a peptide such that it retains reactivity with Crohn's disease sera or tolerogenic activity. Examples of amino acids and amino acids analogs are listed in Gross and Meienhofer, The Peptides: Analysis, Synthesis, Biology, Academic Press, Inc., New York (1983). An amino acid also can be an amino acid mimetic, which is a structure that exhibits substantially the same spatial arrangement of functional groups as an amino acid but does not necessarily have both the α-amino and α-carboxyl groups characteristic of an amino acid.

[0029] A reactive or tolerogenic fragment of an OmpC antigen useful in the invention can be produced or synthesized using methods well known in the art. Such methods include recombinant DNA methods and chemical synthesis methods for production of a peptide. Recombinant methods of producing a peptide through expression of a nucleic acid sequence encoding the peptide in a suitable host cell are well known in the art and are described, for example, in Ausubel et al., *supra*, 1999.

[0030] A reactive or tolerogenic fragment of an OmpC antigen useful in the invention also can be produced by chemical synthesis, for example, by the solid phase peptide synthesis method of Merrifield et al., J. Am. Chem. Soc. 85: 2149 (1964). Standard solution methods well known in the art also can be used to synthesize a reactive or tolerogenic fragment useful in the invention (see, for example, Bodanszky, Principles of Peptide Synthesis, Springer-Verlag, Berlin (1984) and Bodanszky, Peptide Chemistry, Springer-Verlag, Berlin (1993)). A newly synthesized peptide can be purified, for example, by high performance liquid chromatography (HPLC), and can be characterized using, for example, mass spectrometry or amino acid sequence analysis.

[0031] It is understood that limited modifications can be made to an OmpC antigen without destroying its biological function. Similarly, limited modifications can be made to a reactive fragment of an OmpC antigen or a tolerogenic fragment of this antigen without destroying its reactivity or tolerogenic activity. A modification of an antigen disclosed herein that does not destroy its preferential reactivity with IgA antibodies in the sera of patients having Crohn's disease or a modification of an antigen disclosed herein that does not destroy tolerogenic activity is within the definition of an OmpC antigen. Similarly, a modification of a reactive fragment of an antigen disclosed herein that does not destroy its ability to form a complex with IgA antibodies in the sera of a patient having Crohn's disease is within the definition of a reactive fragment. Furthermore, a modification of a tolerogenic fragment of an OmpC antigen that does not destroy its ability to produce a decreased immunological response is within the definition of a tolerogenic fragment of an OmpC

antigen. A modification can be, for example, an addition, deletion, or substitution of amino acid residues; substitution of a compound that mimics amino acid structure or function; or addition of chemical moieties such as amino or acetyl groups. The activity of a modified OmpC antigen or a modified fragment of an OmpC antigen can be assayed, for example, using one of the assays for reactivity or tolerogenic activity disclosed herein.

[0032] A particularly useful modification confers increased stability. Incorporation of one or more D-amino acids is a modification useful in increasing stability of a OmpC antigen or fragment thereof. Similarly, deletion or substitution of lysine can increase stability by protecting against degradation. For example, such a substitution can increase stability and, thus, bioavailability of an OmpC antigen or a tolerogenic fragment thereof, provided that the substitution does not significantly impair reactivity or tolerogenic activity.

[0033] The invention further provides a method of diagnosing Crohn's disease by determining the presence or absence of IgA anti-OmpC antibodies in a sample from the subject and the presence or absence of IgA anti-*Saccharomyces cerevisiae* antibodies (ASCA) in a sample from the subject, where the presence of IgA anti-OmpC antibodies or the presence of IgA ASCA in the sample from the subject each independently indicates that the subject has Crohn's disease. The presence of IgA ASCA can be determined, for example, by reactivity with purified yeast cell wall phosphopeptidomannan (PPM), which can be prepared, for example, from ATCC strain #38926. Methods for determining the presence of IgA ASCA are exemplified herein in Example III. As used herein, the "presence of IgA ASCA" means IgA reactivity against *S. cerevisiae* that is greater than 20% of the reactivity given by reference (known positive) Crohn's disease sera.

[0034] Anti-*Saccharomyces cerevisiae* antibodies (ASCA) are characteristically elevated in patients having Crohn's disease although the nature of the *S. cerevisiae* antigen supporting the specific antibody response in CD is unknown (Sendid et al., Clin. Diag. Lab. Immunol., 3:219-226 (1996)). These antibodies may represent a response against yeasts present in common food or drink or a response against yeasts that colonize the gastrointestinal tract. Studies with periodate oxidation have shown that the epitopes recognized by ASCA in CD patient sera contain polysaccharides. Oligomannosidic epitopes are shared by a variety of organisms including different yeast strains and genera, filamentous fungi, viruses, bacteria and human glycoproteins. Thus, the mannose-induced antibody responses in CD may represent a response against a pathogenic yeast organism or may represent a response against a cross-reactive oligomannosidic epitope present, for example, on a human glycoprotein autoantigen. Regardless of the nature of the antigen, elevated levels of serum ASCA are a differential marker for Crohn's disease, with only low levels of ASCA reported in UC patients (Sendid et al., *supra,* 1996).

[0035] IgA ASCA can be detected using an antigen specific for ASCA, which is any antigen or mixture of antigens that is bound specifically by ASCA. Although ASCA antibodies were initially characterized by their ability to bind *S. cerevisiae,* those of skill in the art will understand that an antigen specific for ASCA can be obtained from *S. cerevisiae,* or can be obtained from a variety of other sources so long as the antigen is capable of binding specifically to ASCA antibodies. Accordingly, exemplary sources of an antigen specific for ASCA contemplated for use in the methods of the invention include whole killed yeast cells, such as from the genera *Saccharomyces and Candida,* yeast cell wall phosphopeptidomannan (PPM), oligomannosides, neoglycolipids, anti-ASCA idiotypic antibodies, and the like. As described above, different species and strains of yeast, including *Saccharomyces,* can be used as an antigen specific for ASCA in the methods provided herein. For example, *S. cerevisiae* strain Su1, Su2, CBS 1315 or BM 156, or *Candida albicans* strain VW32, can be used as an antigen specific for ASCA in the methods of the invention.

[0036] Preparations of yeast cell wall mannans, or phosphopeptidomannans (PPM), are also contemplated herein as antigens specific for ASCA. These water soluble surface antigens can be prepared by appropriate extraction techniques, including autoclaving as described in Example III or can be obtained commercially (see Lindberg et al., Gut 33:909-913 (1992)). The acid stable fraction of yeast cell wall PPM also can be useful in the methods of the invention (Sendid et al., *supra,* 1996). An exemplary PPM for use in diagnosing clinical subtypes of Crohn's disease is derived from S. *cerevisiae* strain ATCC #38926.

[0037] Purified oligosaccharide antigens, such as oligomannosides specific for ASCA, also are contemplated for use in the methods of the invention. For use herein, the purified oligomannoside antigens are preferably converted into neoglycolipids as described in Faille et al., Eur. J. Microbiol. Infect. Dis. 11:438-446 (1992). One skilled in the art understands that the reactivity of such an oligomannoside antigen with ASCA can be optimized by varying the mannosyl chain length (Frosh et al., Proc. Natl. Cad. Sci. USA, 82:1194-1198 (1985)); the anomeric configuration (Fukazawa et al., *In* E. Kurstak (ed.), Immunology of Fungal Disease, Marcel Dekker Inc., New York, pp. 37-62 (1989); Nishikawa et al, Microbiol. Immunol., 34:825-840 (1990); Poulain et al., Eur. J. Clin. Microbiol, 23:46-52 (1993); Shibata et al., Arch. Biochem. Biophys., 243:338-348 (1985); and Trinel et al., Infect. Immun., 60:3845-3851 (1992)); or the position of the linkage (Kikuchi et al., Planta, 190:525-535 (1993)).

[0038] An oligomannoside antigen specific for ASCA can include the mannotetraose Man(1-3)Man(1-2)Man(1-2) Man, and can be purified from PMM as described in Faille et al., *supra,* 1992. An exemplary neoglycolipid for use in the methods of the invention can be constructed by releasing the oligomannoside from its respective PPM and subsequently coupling the released oligomannoside to 4-hexadecylaniline or the like.

[0039] The invention additionally provides a method of diagnosing Crohn's disease by determining the presence or absence of IgA anti-OmpC antibodies in a sample from the subject and the presence or absence of IgA anti-I-2 polypeptide antibodies in a sample from the subject, where the presence of IgA anti-OmpC antibodies or the presence of IgA anti-I-2 polypeptide antibodies in the sample from the subject each independently indicates that the subject has Crohn's disease. In one embodiment, the presence of IgA anti-I-2 polypeptide antibodies is determined by IgA reactivity against an I-2 polypeptide having substantially the amino acid sequence of SEQ ID NO: 3.

[0040] The methods of the invention relate to determining the presence or absence of IgA anti-I-2 polypeptide antibodies in a sample from a subject. As used herein, the "presence of IgA anti-I-2 polypeptide antibodies" or "IgA anti-I-2 antibodies" means IgA reactivity against an I-2 polypeptide that is greater than two standard deviations above the IgA anti-I-2 mean reactivity of control (normal) sera analyzed under the same conditions.

[0041] As used herein, the term "I-2 polypeptide" means a polypeptide having substantially the same amino acid sequence as the microbial I-2 polypeptide (SEQ ID NO: 3) shown in Figure 6. The microbial I-2 polypeptide (SEQ ID NO: 3) is a polypeptide of 100 amino acids sharing some similarity to bacterial transcriptional regulators, with the greatest similarity in the amino-terminal 30 amino acids. The I-2 encoding nucleic acid (SEQ ID NO: 2) is differentially present in involved Crohn's disease tissue, as compared to mucosa macroscopically free of disease. An I-2 polypeptide, or reactive fragment thereof, can be prepared, for example, using recombinant methods as set forth in Example IV.

[0042] An I-2 polypeptide having substantially the same amino acid sequence as SEQ ID NO: 3 can be the naturally occurring I-2 polypeptide (SEQ ID NO: 3) or a related polypeptide having substantial amino acid sequence similarity to this sequence. Such related polypeptides include isotype variants or homologs of the amino acid sequence shown in Figure 6. As used herein, the term I-2 polypeptide generally describes polypeptides generally having an amino acid sequence with greater than about 50% identity, preferably greater than about 60% identity, more preferably greater than about 70% identity, and can be a polypeptide having greater than about 80%, 90%, 95%, 97%, or 99% amino acid sequence identity with SEQ ID NO: 3, said amino acid identity determined with CLUSTALW using the BLOSUM 62 matrix with default parameters.

[0043] Further provided by the invention is a method of diagnosing Crohn's disease in a subject by determining the presence or absence of IgA anti-OmpC antibodies in a sample from the subject; determining the presence or absence of IgA ASCA in a sample from the subject; and determining the presence or absence of IgA anti-I-2 polypeptide antibodies in a sample from the subject, where the presence of the IgA anti-OmpC antibodies, the presence of IgA ASCA or the presence of IgA anti-I-2 polypeptide antibodies each independently indicates that the subject has Crohn's disease.

[0044] Previous studies have shown ANCA reactivity in a small portion of patients with Crohn's disease, although these antibodies are elevated more frequently in patients with ulcerative colitis. An assay for ulcerative colitis based on pANCA or pANCA and anti OmpC IgG levels, respectively, was disclosed in Cohavi et al. (Infect. Immun. 68, 1542-1548) and US 6,033,864. The reported prevalence in CD varies from 0 to 43% with most studies reporting that 10 to 30% of CD patients express ANCA (see, for example, Saxon et al., J. Allergy Clin. Immunol. 86:202--210 (1990); Cambridge et al., Gut 33:668-674 (1992); Pool et al., Gut 3446-50 (1993); and Brokroelofs et al., Dio. Dis. Sci. 39: 545-549 (1994).

[0045] In a method of the invention, the presence or absence of pANCA can be optionally determined in the subject, for example, by reactivity with fixed neutrophil. As used herein, the term "perinuclear anti-neutrophil cytoplasmic antibody" is synonymous with "pANCA" and refers to an antibody that reacts specifically with a neutrophil to give perinuclear to nuclear staining or cytoplasmic staining with perinuclear highlighting. A method for determining the presence of pANCA in a subject is exemplified herein in Example V.

[0046] The invention further provides the use of an OmpC antigen, or tolerogenic fragment thereof for preparing a medicament to induce tolerance in a patient with Crohn's disease. An OmpC antigen useful for a medicament for inducing tolerance can include, for example, the sequence SEQ ID NO: 1.

[0047] As used herein, the term "effective dose" means the amount of an OmpC antigen, or a tolerogenic fragment thereof, useful for inducing tolerance in a patient having Crohn's disease. For induction of oral tolerance, for example, multiple smaller oral doses can be administered or a large dose can be administered. Such doses can be extrapolated, for example, from the induction of tolerance in animal models (see, for example, Trentham et al., Science 261: 1727-1730 (1993)).

[0048] Various molecules are known in the art to cause, promote or enhance tolerance, and an OmpC antigen or tolerogenic fragment thereof can be combined, if desired, with a tolerogizing molecule. See, for example, U.S. Patent No. 5,268,454, and citations therein. As used herein, the term "tolerogizing molecule" means a molecule, compound or polymer that causes, promotes or enhances tolerogenic activity when combined with an OmpC antigen or fragment thereof. A tolerogizing molecule can be optionally conjugated to an OmpC antigen and can be, for example, polyethylene glycol. Such molecules are well known in the art (see, for example, U.S. Patent No. 5,268,454, *supra*).

[0049] An effective dose of an OmpC antigen, or a fragment thereof, for inducing tolerance can be administered by methods well known in the art. Oral tolerance is well-recognized in the art as a method of treating autoimmune disease

(see, for example, Weiner, Hospital Practice, pp. 53-58 (Sept. 15, 1995)). For example, orally administered autoantigens suppress several experimental autoimmune models in a disease- and antigen-specific fashion; the diseases include experimental autoimmune encephalomyelitis, uveitis, and myasthenia, collagen- and adjuvant-induced arthritis, and diabetes in the NOD mouse (see, for example, Weiner et al., Ann. Rev. Immunol. 12:809-837 (1994)). Furthermore, clinical trials of oral tolerance have produced positive results in treating multiple sclerosis, rheumatoid arthritis and uveitis. Modes of administration include parenteral administration and subcutaneous injection (Johnson, Ann. Neurology 36(suppl.):S115-S117 (1994)).

[0050] The term "tolerogenic fragment," as used in reference to an OmpC antigen, means a peptide or polypeptide portion of the antigen that has tolerogenic activity as defined by its ability either alone, or in combination with another molecule, to produce a decreased immunological response. Thus, a tolerogenic fragment of an OmpC antigen is a peptide or polypeptide that has substantially the same amino acid sequence as a portion of SEQ ID NO: 1 and tolerogenic activity as defined by its ability either alone, or in combination with another molecule, to produce a decreased immunological response. A tolerogenic fragment of an OmpC antigen can have from about three amino acids to about 90 amino acids. A tolerogenic fragment of an OmpC antigen can have, for example, at least 5, 8, 10, 12, 15, 18, 20 or 25 amino acids. For example, a tolerogenic fragment of an OmpC antigen can have from five to fifty amino acids, from eight to fifty amino acids, or from ten to fifty amino acids. More preferably, a tolerogenic fragment has from eight to twenty amino acids or from.ten to twenty amino acids. Most preferably, a tolerogenic fragment has from twelve to twenty amino acids or from fifteen to twenty amino acids.

[0051] A tolerogenic fragment of an OmpC antigen can be identified using one of a variety of assays, including *in vitro* assays such as T-cell proliferation or cytokine secretion assays and *in vivo* assays such as the induction of tolerance in murine models of inflammatory bowel disease. T-cell proliferation assays, for example, are well recognized in the art as predictive of tolerogenic activity (see, for example, Miyahara et al., Immunol. 86:110-115 (1995) or Lundin et al, J. Exp. Med. 178:187-196 (1993)). A T-cell proliferation assay can be performed by culturing T-cells with irradiated antigen-presenting cells, such as normal spleen cells, in microtiter wells for 3 days with varying concentrations of the fragment to be assayed; adding ^{3}H-thymidine; and measuring incorporation of ^{3}H-thymidine into DNA. In such an assay, a fragment of an OmpC antigen can be tested for activity, for example, at concentrations of 20 μg/ml and 40 μg/ml.

[0052] A tolerogenic fragment of an OmpC antigen can be identified using a T-cell cytokine secretion assay known in the art. For example, T cells can be cultured with irradiated antigen-presenting cells in microtiter wells with varying concentrations of the fragment of interest and, after three days, the culture supernatants can be assayed for IL-2, IL-4 or IFN-γ as described in Czerinsky et al., Immunol. Rev. 119:5-22 (1991).

[0053] Primary T-cells for use in a T-cell proliferation assay or cytokine secretion assay, for example, can be isolated from lamina propria or peripheral blood. In addition, a convenient source of T-cells for use in an *in vitro* assay for tolerogenic activity can be a T-cell line established from an IBD patient such as a Crohn's disease patient, from a murine model of IBD or from a healthy animal immunized with an OmpC antigen of the invention. A preferred source of T-cells for use in identifying a tolerogenic fragment of an OmpC antigen is a Crohn's disease patient.

[0054] A T-cell line can be established from a patient with CD or UC, for example, by culturing T lymphocytes with about 1 μg/ml recombinant OmpC antigen or OmpC fusion protein, in the presence of low concentrations of growth-supporting IL-2 (about 10 μg/ml). A T-cell line can be established by culturing T lymphocytes with antigen-presenting cells and feeding the cells on an alternating four to five day cycle with either IL-2 and an OmpC antigen, or IL-2 alone, as described in Nanda et al., J. Exp. Med. 176:297-302 (1992). A cell line that develops into a reliably proliferating cell line dependent on the presence of an OmpC antigen is particularly useful in identifying tolerogenic fragments. The establishment of T-cell lines from small intestinal mucosa is described, for example, in Lundin et al., *supra,* 1993. T cell lines dependent upon the presence of an OmpC antigen and useful for identifying tolerogenic fragments of an OmpC antigen can be prepared similarly.

[0055] A tolerogenic fragment also can be identified by its ability to induce tolerance *in vivo,* as indicated by a decreased immunological response, which can be a decreased T-cell response, such as a decreased proliferative response or cytokine secretion response as described above, or a decreased antibody titer to the antigen. A neonatal or adult mouse can be tolerized with a fragment of an OmpC antigen, and a T-cell response or anti-OmpC antibody titer can be assayed after challenging by immunization. For example, a neonatal mouse can be tolerized within 48 hours of birth by intraperitoneal administration of about 100 μg of a fragment of an OmpC antigen emulsified with incomplete Freund's adjuvant and subsequently immunized with I-2 polypeptide at about 8 weeks of age (see, for example, Miyahara, *supra,* 1995). An adult mouse can be tolerized intravenously with about 0.33 mg of a fragment of an OmpC antigen, administered daily for three days (total dose 1 mg), and immunized one week later with an OmpC antigen. A decreased T-cell response, such as decreased proliferation or cytokine secretion, which indicates tolerogenic activity, can be measured using T-cells harvested 10 days after immunization. In addition, a decreased anti-OmpC antibody titer, which also indicates tolerogenic activity, can be assayed using blood harvested 4-8 weeks after immunization. Methods for assaying a T-cell response or anti-OmpC antigen antibody titer are described above and are well known in the art.

[0056] A tolerogenic fragment of an OmpC antigen also can be identified using a murine model of inflammatory bowel disease. Neonatal or adult mice having IBD-like disease can be tolerized with a fragment of an OmpC antigen as described above, and the T-cell response or anti-OmpC antibody titer assayed. A decreased T-cell response or decreased antibody titer to the antigen indicates a decreased immunological response and, thus, serves to identify a tolerogenic fragment of an OmpC antigen. In addition, a tolerogenic fragment of an OmpC antigen can be identified by the ability to reduce the frequency, time of onset or severity of colitis in a murine model of IBD.

[0057] Several well-accepted murine models of inflammatory bowel disease can be useful in identifying a tolerogenic fragment of an OmpC antigen. For example, mice with targeted disruption of the gene encoding the alpha subunit of the G-protein Gi2, is a well known model exhibiting features of human bowel disease (Hornquist et al., J. Immunol. 158:1068-1077 (1997); Rudolph et al., Nat. Genet. 10:143-150 (1995)). Mice deficient in IL-10 as described in Kühn et al., Cell 75:263-274 (1993), and mice deficient in IL-2 as described in Sadlack et al., Cell 75:253-261 (1993), also have colitis-like disease and are useful in identifying a tolerogenic fragment of an OmpC antigen. Furthermore, mice with mutations in T cell receptor (TCR) α, TCR β, TCR $\beta \times \delta$, or the class II major histocompatiblility complex (MHC) as described in Mombaerts et al., Cell 75:275-282 (1993), develop inflammatory bowel disease and, thus, are useful in identifying a tolerogenic fragment of an OmpC antigen. Similarly, a fragment can be assayed for tolerogenic activity in a SCID mouse reconstituted with CD45RB CD4+ T-cells, which is a well-accepted model of inflammatory bowel disease, as described in Powrie et al., Immunity 1:553-562 (1994). Additional animal models of IBD also are well known in the art (see, for example, Podolsky, Acta Gastroenterol. Belg. 60:163-165 (1997); and Bregenholt et al., APMIS 105: 655-662 (1997)). Thus, a tolerogenic fragment of an OmpC antigen can be readily identified by an *in vitro or in vivo* assay disclosed herein or by another assay well known in the art.

[0058] A reactive or tolerogenic fragment of an OmpC antigen can be identified by screening a large collection, or library, of peptides of interest or random peptides for reactivity or tolerogenic activity. For example, a panel of peptides spanning the entire sequence of an OmpC antigen can be screened for reactivity or tolerogenic activity as described above. Such a panel can be a panel of 15-mer peptides spanning the sequence of the OmpC antigen (SEQ ID NO: 1), each overlapping by three or five residue shifts using the Mimotope cleavable pin technology (Cambridge Research Biochemicals, Wilmington, DE), as described by Geysen et al., Science 235:1184 (1987). The panel is subsequently screened for reactivity or tolerogenic activity using one of the assays described above (see, for example, Miyahara et al., *supra*, 1995). A library of peptides to be screened also can be a population of peptides related in amino acid sequence to SEQ ID NO: 1 but having one or more amino acids that differ from SEQ ID NO: 1.

[0059] Additional peptides to be screened include, for example, tagged chemical libraries of peptides and peptidomimetic molecules. Peptide libraries also comprise those generated by phage display technology. Phage display technology includes the expression of peptide molecules on the surface of phage as well as other methodologies by which a protein ligand is or can be associated with the nucleic acid which encodes it. Methods for production of phage display libraries, including vectors and methods of diversifying the population of peptides which are expressed, are well known in the art (see, for example, Smith and Scott, Methods Enzymol. 217:228-257 (1993); Scott and Smith, Science 249: 386-390 (1990); and Huse, WO 91/07141 and WO 91/07149). These or other well known methods can be used to produce a phage display library which can be screened, for example, with one of the disclosed assays for reactivity or tolerogenic activity. If desired, a population of peptides can be assayed for activity en masse. For example, to identify a reactive fragment of an OmpC antigen, a population of peptides can be assayed for the ability to form a complex with patient sera containing IgA anti-OmpC antigen reactivity; the active population can be subdivided, and the assay repeated in order to isolate the reactive fragment from the population.

[0060] A reactive or tolerogenic fragment of an OmpC antigen also can be identified by screening, for example, fragments of the polypeptide produced by chemical or proteolytic cleavage. Methods for chemical and proteolytic cleavage and for purification of the resultant protein fragments are well known in the art (see, for example, Deutscher, Methods in Enzymology, Vol. 182, "Guide to Protein Purification," San Diego: Academic Press, Inc. (1990)). For example, a chemical such as cyanogen bromide or a protease such as trypsin, chymotrypsin, V8 protease, endoproteinase Lys-C, endoproteinase Arg-C or endoproteinase Asp-N can be used to produce convenient fragments of an OmpC antigen that can be screened for reactivity or tolerogenic activity using one of the assays disclosed herein.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

PURIFICATION OF OmpC

[0061] This example describes purification of OmpC using spheroplast lysis.

[0062] $OmpF^{-/-}/OmpA^{-/-}$ mutant *E. coli* were inoculated from a glycerol stock into 10-20ml of Luria Bertani broth supplemented with 100 µg/ml streptomycin (LB-Strep), and cultured vigorously at 37°C for ~8 hours to log phase. This starter culture was used to inoculate one liter of LB-strep media, and the 1 L culture grown for less than 15 hours.

[0063] The cells were harvested by centrifugation (JS-4.2, 4K/15min/4°C). If necessary, cells were washed twice with 100 ml of ice cold 20 mM Tris-Cl pH 7.5. The cells were subsequently resuspended in ice cold spheroplast forming buffer (20 mM Tris-Cl pH 7.5, 20% sucrose, 0.1 M EDTA pH 8.0, 1 mg/ml lysozyme), after which the resuspended cells were incubated on ice for 20 minutes to 2 hours with occasional mixing by inversion.

[0064] If required, the spheroplasts were centrifuged (JA-17, 5.5k/10min/4°C) and resuspended in a smaller volume of spheroplast forming buffer (SFB). The spheroplast pellet was optionally frozen prior to resuspension in order to improve lysis efficiency. Hypotonic buffer was avoided in order to avoid bursting the spheroplasts and releasing chromosomal DNA, which significantly decreases the efficiency of lysis.

[0065] The spheroplast preparation was diluted 14-fold into ice cold 10 mM Tris-Cl pH 7.5, 1 mg/ml DNase-I, and vortexed vigorously. The preparation was sonicated on ice 4 x 30 seconds at 50% power at setting 4, with a pulse "On time" of 1 second, without foaming or overheating the sample.

[0066] Cell debris was pelleted by centrifugation (JA-17, 5-10K/10min/4°C), and the supernatant removed and clarified by centrifugation a second time (10K/10 min/4°C). The supernatant was removed without collecting any part of the pellet, and placed into ultra centrifuge tubes. The tubes were filled to 1.5 millimeter from top with 20 mM Tris-Cl pH 7.5.

[0067] The membrane preparation was pelleted by ultra centrifugation at 100,000 g (35K/1 hour/4°C in Beckman SW 60 swing bucket rotor). The pellet was resuspended by homogenizing into 20 mM Tris-Cl pH 7.5 using a 1 ml blue pipette tip and squirting the pellet closely before pipetting up and down for approximately ~10 minutes per tube.

[0068] In a 15 ml screw cap tube filled with 4 mls, the material was extracted for 1 hour in 20 mM Tris-Cl pH 7.5 with 1% SDS, with rotation at 37°C. The preparation was transferred to ultra centrifugation tubes, and the membrane pelleted at 100,000g (35K/1 hour/4°C in Beckman SW 60). The pellet was resuspended by homogenizing into 20 mM Tris-Cl pH 7.5 as before. The membrane preparation was optionally left at 4°C overnight.

[0069] OmpC was extracted for 1 hour with rotation at 37°C in 20 mM Tris-Cl pH 7.5, 3%SDS, and 0.5 M NaCl (SDS will precipitate if kept below 37°C). The material was transferred to ultra centrifugation tubes, and the membrane pelleted by centrifugation at 100,000g (35K/1 hour/30°C in Beckman SW 60). Lower temperatures were avoided since further cooling will result in extracted protein salting out of solution.

[0070] The supernatant containing extracted OmpC was then dialyzed against more than 10,000 volumes to eliminate high salt content. SDS was removed by detergent exchange against 0.2% Triton. Triton was removed by further dialysis against 50 mM Tris-Cl.

[0071] Purified OmpC, which functions as a porin in its trimeric form, is characterized as follows when analyzed by SDS-PAGE. Electrophoresis at room temperature resulted in a ladder of ~100 kDa, ~70 kDa, and ~30 kDa bands. Heating for 10-15 minutes at 65-70°C partially dissociated the complex and resulted in only dimers and monomers (~70 kDa and ~30 kDa bands). Boiling for 5 minutes resulted in monomers of 38 kDa.

EXAMPLE II

ANTI-IgA OmpC ELISA ASSAYS

[0072] This example describes an ELISA assay for direct detection of IgA anti-OmpC antibodies in patient sera.

[0073] The OmpC direct ELISA assay was performed as follows. Plates (Immulon 3, DYNEX Technologies, Chantilly, VA) were coated overnight at 4 C with 100 µl/well of OmpC prepared as described above at 0.25 µg/ml in borate buffered saline, pH 8.5. After three washes in 0.05% Tween 20 in phosphate buffered saline (PBS), the plates were blocked with 150 µl/well of 0.5% bovine serum albumin in PBS, pH 7.4 (BSA-PBS) for 30 minutes at room temperature (RT). The blocking solution was then discarded, and 100 µl/well of serum from Crohn's disease patients, ulcerative colitis patients and normal controls diluted 1:100 was added and incubated for 2 hours at room temperature. After washing the plates as before, alkaline phosphatase-conjugated indicator antibody (goat anti-human IgA (α-chain specific) from Jackson ImmunoResearch, West Grove, PA) was added to the plates at a dilution of 1:1000 in BSA-PBS, and the plates were incubated at room temperature for 2 hours. The plates were subsequently washed three times with 0.05% Tween 20 in phosphate buffered saline, followed by another three washes with Tris buffered normal saline, pH 7.5. Substrate solution (1.5 mg/ml disodium P-nitrophenol phosphate (Amresco; Solon, OH), 2.5 mM $MgCl_2$, 0.01 M Tris, pH 8.6) was added at 100 µl/well, and color was allowed to develop for one hour before the plates were read at 405 nm.

[0074] IgA OmpC positive reactivity was defined as reactivity greater than two standard deviations above the mean reactivity obtained with control (normal) sera analyzed at the same time as the test samples.

EXAMPLE III

IaA ASCA ELISA ASSAY

[0075] This example demonstrates that the presence of IgA *anti-Saccharomyces cerevisiae* antibodies in patient sera can be determined using an ELISA microplate assay.

A. Preparation of yeast cell wall mannan

[0076] Yeast cell wall mannan was prepared as follows and as described in Faille et al. Eur. J. Clin. Microbiol. Infect. Dis. 11:438-446 (1992) and in Kocourek and Ballou et al., J. Bacteriol 100:1175-1181 (1969). A lyophilized pellet of yeast *Saccharomyces uvarum* was obtained from the American Type Culture Collection (#38926). Yeast were reconstituted in 10 ml 2X YT medium, prepared according to Sambrook et al., Molecular Cloning Cold Spring Harbor Laboratory Press (1989). *S. uvarum* were grown for two to three days at 30°C. The terminal *S. uvarum* culture was inoculated on a 2X YT agar plate and subsequently grown for two to three days at 30°C. A single colony was used to inoculate 500 ml 2X YT media, and grown for two to three days at 30°C. Fermentation media (pH 4.5) was prepared by adding 20 gm glucose, 2 gm bacto-yeast extract, 0.25 gm $MgSO_4$ and 2.0 ml 28% H_3PO_4 per liter distilled water. The 500 ml culture was used to inoculate 50 liters of fermentation media, and the culture fermented for three to four days at 37°C.

[0077] *S. uvarum* mannan extract was prepared by adding 50 ml 0.02 M citrate buffer (5.88 gm/l sodium citrate; pH 7.0+/-0.1) to each 100 grams of cell paste. The cell/citrate mixture was autoclaved at 125°C for ninety minutes and allowed to cool. After centrifuging at 5000 rpm for 10 minutes, the supernatant was removed and retained. The cells were then washed with 75 ml 0.02 M citrate buffer and the cell/citrate mixture again autoclaved at 125°C for ninety minutes. The cell/citrate mixture was centrifuged at 5000 rpm for 10 minutes, and the supernatant retained.

[0078] In order to precipitate copper/mannan complexes, an equal volume of Fehling's Solution was added to the combined supernatants while stirring. The complete Fehling's solution was prepared by mixing Fehling's Solution A with Fehling's Solution B in a 1:1 ratio just prior to use. The copper complexes were allowed to settle, and the liquid decanted gently from the precipitate. The copper/mannan precipitate complexes were then dissolved in 6-8 ml 3N HCl per 100 grams yeast paste.

[0079] The resulting solution was poured with vigorous stirring into 100 ml of 8:1 methanol:acetic acid, and the precipitate allowed to settle for several hours. The supernatant was decanted and discarded; then the wash procedure was repeated until the supernatant was colorless, approximately two to three times. The precipitate was collected on a scintered glass funnel, washed with methanol and air dried overnight. On some occasions, the precipitate was collected by centrifugation at 5000 rpm for 10 minutes before washing with methanol and air drying overnight. The dried mannan powder was dissolved in distilled waster, using approximately 5 ml water per gram of dry mannan powder. The final concentration of *S. uvarum* cell wall mannan was approximately 30 µg/ml.

B. Preparation of S. uvarum *mannan ELISA plates*

[0080] *S. uvarum* cell mannan ELISA plates were saturated with antigen as follows. Purified *S. uvarum* mannan prepared as described above was diluted to a concentration of 100 µg/ml with phosphate buffered saline/0.2% sodium azide (PBS-N3). Using a multi-channel pipettor, 100 µl of 100 µg/ml *S. uvarum* mannan was added per well of a Costar 96-well hi-binding plate (catalogue number 3590; Costar Corp., Cambridge, MA). The antigen was allowed to coat the plate at 4° C for a minimum of 12 hours. Each lot of plates was compared to a previous lot before use. Plates were stored at 2-8° C for up to one month.

C. Analysis of patient sera

[0081] Patient sera were analyzed in duplicate for anti-IgG or anti-IgA reactivity. Microtiter plates saturated with antigen as described above were incubated with phosphate buffered saline/0.05% Tween-20 for 45 minutes at room temperature to inhibit nonspecific antibody binding. Patient sera were subsequently added at a dilution of 1:80 for IgA and incubated for 1 hour at room temperature. Wells were washed three times with PBS/0.05% Tween-20. Then a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-human IgA (Jackson Immunoresearch, Westgrove, PA) was added, and the microtiter plates incubated for 1 hour at room temperature. A solution of *p*-nitrophenol phosphate in diethanolamine substrate buffer was added, and color development allowed to proceed for 10 minutes. Absorbance at 405 nm was analyzed using an automated EMAX plate reader (Molecular Devices, Sunnyvale, CA).

[0082] Standard binding of pooled sera from patients with an established diagnosis of Crohn's disease was used as a standard reference for binding and set to be 100 ELISA units. Results with test patient sera were expressed as a

percentage of the standard binding of the reference CD sera. ASCA-positivity was defined as IgA ASCA reactivity that was greater than 20% of the reference CD sera.

EXAMPLE IV

IgA I-2 ELISA

[0083] This example demonstrates that the I-2 polypeptide can be determined using ELISA analysis.

A. GST-I-2 fusion protein

[0084] The full-length I-2 encoding nucleic acid sequence (SEQ ID NO: 3) was cloned into the GST expression vector pGEX. After expression in *E. coli*, the protein was purified on a GST column. The purified protein was shown to be of the expected molecular weight by silver staining, and had anti-GST reactivity upon western analysis.

B. ELISA analysis

[0085] Human IgA antibodies that bind the I-2 polypeptide (SEQ ID NO: 3) were detected by direct ELISA assays essentially as follows. Plates (Immulon 3; DYNEX Technologies; Chantilly, VA) were coated overnight at 4°C with 100 μl/well GST-I-2 fusion polypeptide (5 μg/ml in borate buffered saline, pH 8.5). After three washes in 0.05% Tween 20 in phosphate buffered saline (PBS), the plates were blocked with 150 μl/well of 0.5% bovine serum albumin in PBS, pH 7.4 (BSA-PBS) for 30 minutes at room temperature. The blocking solution was then replaced with 100 μl/well of Crohn's disease or normal control serum, diluted 1:100. The plates were then incubated for 2 hours at room temperature and washed as before. Alkaline phosphatase conjugated secondary antibody [goat anti-human IgA (α-chain specific), Jackson ImmunoResearch, West Grove, PA] was added to the IgA plates at a dilution of 1:1000 in BSA-PBS. The plates were incubated for 2 hours at room temperature before washing three times with 0.05% Tween 20/PBS followed by another three washes with Tris buffered normal saline, pH 7.5. Substrate solution (1.5 mg/ml disodium P-nitrophenol phosphate (Aresco; Solon, OH) in 2.5 mM $MgCl_2$, 0.01 M Tris, pH 8.6) was added at 100 μl/well, and color allowed to develop for one hour. The plates were then analyzed at 405 nm.

[0086] IgA I-2 positive reactivity was defined as reactivity greater than two standard deviations above the mean reactivity obtained with control (normal) sera analyzed at the same time as the test samples.

EXAMPLE V

ELISA AND INDIRECT IMMUNOFLUORESCENCE FOR DETERMINING pANCA STATUS

[0087] This example describes methods for determining the pANCA status of a subject.

A. Presence of ANCA was determined by fixed neutrophil ELISA

[0088] A fixed neutrophil enzyme-linked immunosorbent assay was used to detect ANCA as described in Saxon et al., J. Allergy Clin. Immunol. 86:202-210 (1990), and all samples were analyzed in a blinded fashion. Microtiter plates were coated with 2.5×10^5 neutrophils per well and treated with 100% methanol to fix the cells. Cells were incubated with 0.25% bovine serum albumin (BSA) in phosphate-buffered saline to block nonspecific antibody binding. Next, control and coded sera were added at a 1:100 dilution to the bovine serum/phosphate-buffered saline blocking buffer. Alkaline phosphatase conjugated goat $F(ab')_2$ anti-human immunoglobulin G (γ-chain specific) antibody (Jackson Immunoresearch Labs, Inc., West Grove, PA) was added at a 1:1000 dilution to label neutrophil bound antibody. A *p*-nitrophenol phosphate substrate solution was added and color development was allowed to proceed until absorbance at 405 nm in the positive control wells was 0.8-1.0 optical density units greater than the absorbance in blank wells.

B. Indirect immunofluorescence assay for determination of ANCA staining pattern

[0089] Indirect immunofluorescent staining was performed on samples that were ANCA-positive by ELISA to determine whether the predominant staining pattern was perinuclear (pANCA) or cytoplasmic (cANCA). Glass slides containing approximately 100,000 neutrophils per slide were prepared by cytocentrifugation (Shandon Cytospin, Cheshire, England) and they were fixed in 100% methanol, air-dried, and stored at -20°C. The fixed neutrophils were incubated with human sera were diluted (1:20), and the reaction was visualized with fluorescein-labeled $F(ab')_2 \cdot \gamma$ chain-specific antibody as described in Saxon et al., *supra*, 1990. The slides were examined using an epifluorescence-equipped

Olympus BH-2 microscope (Olympus, Lake Success, NY).

[0090] pANCA positivity was defined as a perinuclear staining pattern combined with ELISA reactivity greater than two standard deviations above the mean reactivity obtained with control (normal) sera analyzed at the same time as the test samples.

SEQUENCE LISTING

[0091]

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The Regents of the University of California

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Claims

1. A method of diagnosing Crohn's disease in a subject, comprising determining the presence or absence of IgA anti-OmpC antibodies in a sample from said subject, where the presence of said IgA anti-OmpC antibodies indicates that said subject has Crohn's disease.

2. An *in vitro* method of diagnosing Crohn's disease in a subject, comprising the steps of:

   (a) contacting a sample from a subject suspected of having inflammatory bowel disease with an OmpC antigen, or reactive fragment thereof, under conditions suitable to form a complex of the OmpC antigen, or reactive fragment thereof, and IgA antibody to the OmpC antigen;

   (b) contacting said complex with an anti-IgA antibody; and

   (c) detecting the presence or absence of IgA anti-OmpC antibodies, where the presence of said IgA anti-OmpC antibodies in said subject indicates that said subject has Crohn's disease.

3. The method of claim 2, wherein said OmpC antigen is a polypeptide having at least 80% amino acid identity with the amino acid sequence of SEQ ID NO:1.

4. The method of claim 2, wherein IgA anti-OmpC antibodies are detected with an enzyme-linked immunosorbent assay.

5. The method of claim 2, further comprising determining the presence or absence of IgA anti-Saccharomyces cerevisiae antibodies (ASCA) in said subject, wherein the presence of IgA anti-OmpC antibodies or the presence of IgA ASCA in said subject each independently indicates that said subject has Crohn's disease.

6. The method of claim 5, wherein the presence of IgA ASCA is determined by reactivity with purified yeast cell wall phosphopeptidomannan (PPM).

7. The method of claim 6, wherein said yeast cell wall PPM is prepared from ATCC strain #38926.

8. The method of claim 2, further comprising determining the presence or absence of IgA anti-I-2 polypeptide antibodies in said subject, wherein the presence of IgA anti-OmpC antibodies or the presence of IgA anti-I-2 polypeptide antibodies in said subject each independently indicates that said subject has Crohn's disease.

9. The method of claim 8, wherein the presence of IgA anti-I-2 polypeptide antibodies is determined by IgA reactivity with an I-2 polypeptide having greater than about 80% amino acid sequence identity with the amino acid sequence of SEQ ID NO:3.

10. A method of diagnosing Crohn's disease in a subject, comprising the steps of:

(a) determining the presence or absence of IgA anti-OmpC antibodies in a sample from said subject;

(b) determining the presence or absence of IgA ASCA in a sample from said subject;

(c) determining the presence or absence of IgA anti-I-2 polypeptide antibodies in a sample from said subject,

where the presence of said IgA anti-OmpC antibodies the presence of IgA ASCA or the presence of IgA anti-I-2 polypeptide antibodies each independently indicates that said subject has Crohn's disease.

11. The method of claim 10, further comprising determining the presence of absence of perinuclear anti-neutrophil antibodies (pANCA) in said subject.

12. Use of an OmpC antigen, or tolerogenic fragment thereof, for preparing a medicament to induce tolerance in a patient with Crohn's disease.

13. The use of claim 12, wherein said OmpC antigen is a polypeptide having at least 80% amino acid identity with the amino acid sequence of SEQ ID NO:1.

Patentansprüche

1. Verfahren zum Diagnostizieren von Morbus Crohn bei einem Individuum, welches umfasst, die Anwesenheit oder Abwesenheit von IgA-anti-OmpC-Antikörpern in einer Probe von dem Individuum zu bestimmen, wobei die Anwesenheit der IgA-anti-OmpC-Antikörper anzeigt, dass das Individuum Morbus Crohn hat.

2. *In vitro*-Verfahren zum Diagnostizieren von Morbus Crohn bei einem Individuum, welches die Schritte umfasst:

(a) eine Probe von einem Individuum, von welchem vermutet wird, dass es an entzündlicher Darmerkrankung leidet, mit einem OmpC-Antigen oder einem reaktiven Fragment davon in Kontakt zu bringen unter Bedingungen, welche geeignet sind, um einen Komplex des OmpC-Antigens oder des reaktiven Fragments davon und des IgA-Antikörpers gegen das OmpC-Antigen zu bilden;
(b) den Komplex mit einem anti-IgA-Antikörper in Kontakt zu bringen; und
(c) die Anwesenheit oder Abwesenheit von IgA-anti-OmpC-Antikörpern zu detektieren, wobei die Anwesenheit der IgA-anti-OmpC-Antikörper in dem Individuum anzeigt, dass das Individuum Morbus Crohn hat.

3. Verfahren nach Anspruch 2, wobei das OmpC-Antigen ein Polypeptid mit wenigstens 80% Aminosäureidentität mit der Aminosäuresequenz von SEQ ID NO:1 ist.

4. Verfahren nach Anspruch 2, wobei IgA-anti-OmpC-Antikörper mit einem Enzymimmuntest detektiert werden.

5. Verfahren nach Anspruch 2, welches ferner umfasst, die Anwesenheit oder Abwesenheit von IgA-anti-Saccharomyces cerevisiae-Antikörpern (ASCA) in dem Individuum zu bestimmen, wobei die Anwesenheit von IgA-anti-OmpC-Antikörpern oder die Anwesenheit von IgA-ASCA in dem Individuum jeweils unabhängig anzeigt, dass das Individuum Morbus Crohn hat.

6. Verfahren nach Anspruch 5, wobei die Anwesenheit von IgA-ASCA anhand von Reaktivität gegenüber gereinigtem Hefe-Zellwand-Phosphopeptidomannan (PPM) bestimmt wird.

7. Verfahren nach Anspruch 6, wobei das Hefe-Zellwand-PPM aus dem ATCC-Stamm Nr. 38926 hergestellt wird.

8. Verfahren nach Anspruch 2, welches ferner umfasst, die Anwesenheit oder Abwesenheit von IgA-anti-I-2-Polypeptid-Antikörpern in dem Individuum zu bestimmen, wobei die Anwesenheit von IgA-anti-OmpC-Antikörpern oder die Anwesenheit von IgA-anti-I-2-Polypeptid-Antikörpern in dem Individuum jeweils unabhängig anzeigt, dass das Individuum Morbus Crohn hat.

9. Verfahren nach Anspruch 8, wobei die Anwesenheit von IgA-anti-I-2-Polypeptid-Antikörpern anhand von IgA-Re-

aktivität gegenüber einem I-2-Polypeptid, welches mehr als ungefähr 80% Aminosäuresequenzidentität mit der Aminosäuresequenz von SEQ ID NO:3 aufweist, bestimmt wird.

10. Verfahren zum Diagnostizieren von Morbus Crohn bei einem Individuum, welches die Schritte umfasst:

(a) die Anwesenheit oder Abwesenheit von IgA-anti-OmpC-Antikörpern in einer Probe von dem Individuum zu bestimmen;
(b) die Anwesenheit oder Abwesenheit von IgA-ASCA in einer Probe von dem Individuum zu bestimmen;
(c) die Anwesenheit oder Abwesenheit von IgA-anti-I-2-Polypeptid-Antikörpern in einer Probe von dem Individuum zu bestimmen,

wobei die Anwesenheit der IgA-anti-OmpC-Antikörper, die Anwesenheit von IgA-ASCA oder die Anwesenheit von IgA-anti-I-2-Polypeptid-Antikörpern jeweils unabhängig anzeigt, dass das Individuum Morbus Crohn hat.

11. Verfahren nach Anspruch 10, welches ferner umfasst, die Anwesenheit oder Abwesenheit von perinukleären anti-Neutrophilen-Antikörpern (pANCA) in dem Individuum zu bestimmen.

12. Verwendung eines OmpC-Antigens oder eines Toleranz-induzierenden Fragments davon zur Herstellung eines Arzneimittels, um Toleranz in einem Patienten mit Morbus Crohn zu induzieren.

13. Verwendung nach Anspruch 12, wobei das OmpC-Antigen ein Polypeptid mit wenigstens 80% Aminosäureidentität mit der Aminosäuresequenz von SEQ ID NO:1 ist.

Revendications

1. Procédé de diagnostic de la maladie de Crown chez un sujet, comprenant la détermination de la présence ou de l'absence d'anticorps anti-OmpC de type IgA dans un échantillon dudit sujet, où la présence desdits anticorps anti-OmpC de type IgA indique que ledit sujet est atteint de la maladie de Crohn.

2. Procédé de diagnostic *in vitro* de la maladie de Crown chez un sujet comprenant les étapes consistant à :

a) mettre en contact un échantillon d'un sujet chez lequel est suspectée une maladie inflammatoire des intestins, avec un antigène OmpC, ou un fragment réactif de celui-ci, dans des conditions appropriées pour former un complexe entre l'antigène OmpC, ou un fragment réactif de celui-ci et un anticorps anti-antigène OmpC de type IgA ;

b) mettre en contact ledit complexe avec un anticorps anti-IgA ; et

c) détecter la présence ou l'absence d'un anticorps anti-OmpC de type IgA, la présence dudit anticorps anti-OmpC de type IgA chez ledit sujet révélant que ledit sujet est atteint de la maladie de Crohn.

3. Procédé selon la revendication 2 dans lequel ledit antigène OmpC est un polypeptide présentant un degré d'identité d'au moins 80% avec la séquence d'acides aminés de la SEQ ID N°1.

4. Procédé selon la revendication 2 dans lequel les anticorps anti-OmpC de type IgA sont détectés à l'aide d'un test ELISA (enyme-linked immunosorbent assay).

5. Procédé selon la revendication 2, comprenant en outre la détermination de la présence ou de l'absence d'anticorps anti-Saccharomyces cerevisiae (ASCA) de type IgA chez ledit sujet, où la présence d'anticorps anti-OmpC de type IgA ou la présence d'ASCA de type IgA chez ledit sujet indique chacune indépendamment que ledit patient est atteint de la maladie de Crohn.

6. Procédé selon la revendication 5, où la présence d'ASCA de type IgA est déterminée par la réactivité avec le phosphopeptidomannane de la paroi cellulaire de levure purifié (PPM).

7. Procédé selon la revendication 3, où ledit PPM de la paroi cellulaire de levure est préparé à partir de la souche déposée auprès de l'ATCC sous le numéro 38926.

8. Procédé selon la revendication 2, comprenant en outre la détermination de la présence ou de l'absence d'anticorps anti-polypeptide I-2 de type IgA chez ledit sujet, où la présence d'anticorps anti-OmpC de type IgA ou la présence d'anticorps anti-polypeptide I-2 de type IgA chez ledit sujet indique chacune indépendamment que le patient est atteint de la maladie de Crohn.

9. Procédé selon la revendication 8, où la présence d'anticorps anti-polypeptide I-2 de type IgA est déterminée par la réactivité des IgA avec un polypeptide I-2 dont la séquence présente un degré d'identité d'environ 80% avec la séquence d'acides aminés de la SEQ ID N°3.

10. Procédé de diagnostic de la maladie de Crohn chez un sujet, comprenant les étapes consistant à :

a) déterminer la présence ou l'absence d'anticorps anti-OmpC de type IgA dans un échantillon dudit sujet ;

b) déterminer la présence ou l'absence d'ASCA de type IgA dans un échantillon dudit sujet ;

c) déterminer la présence ou l'absence d'anticorps anti-polypeptide I-2 de type IgA dans un échantillon dudit sujet,

où la présence desdits anticorps anti-OmpC de type IgA, la présence d'ASCA de type IgA ou la présence d'anticorps anti-polypeptide I-2 de type IgA indique chacune indépendamment que ledit sujet est atteint de la maladie de Crohn.

11. Procédé selon la revendication 10, comprenant en outre la détermination de la présence ou de l'absence d'anticorps périnucléaires anti-neutrophiles (pANCA) chez ledit sujet.

12. Utilisation de l'antigène OmpC, ou d'un fragment tolérogène de celui-ci, pour la préparation d'un médicament destiné à induire une tolérance chez un patient atteint de la maladie de Crohn.

13. Utilisation selon la revendication 12, où ledit antigène OmpC est un polypeptide possédant une identité de séquence d'au moins 80% avec la séquence d'acides aminés de la SEQ ID N°1.

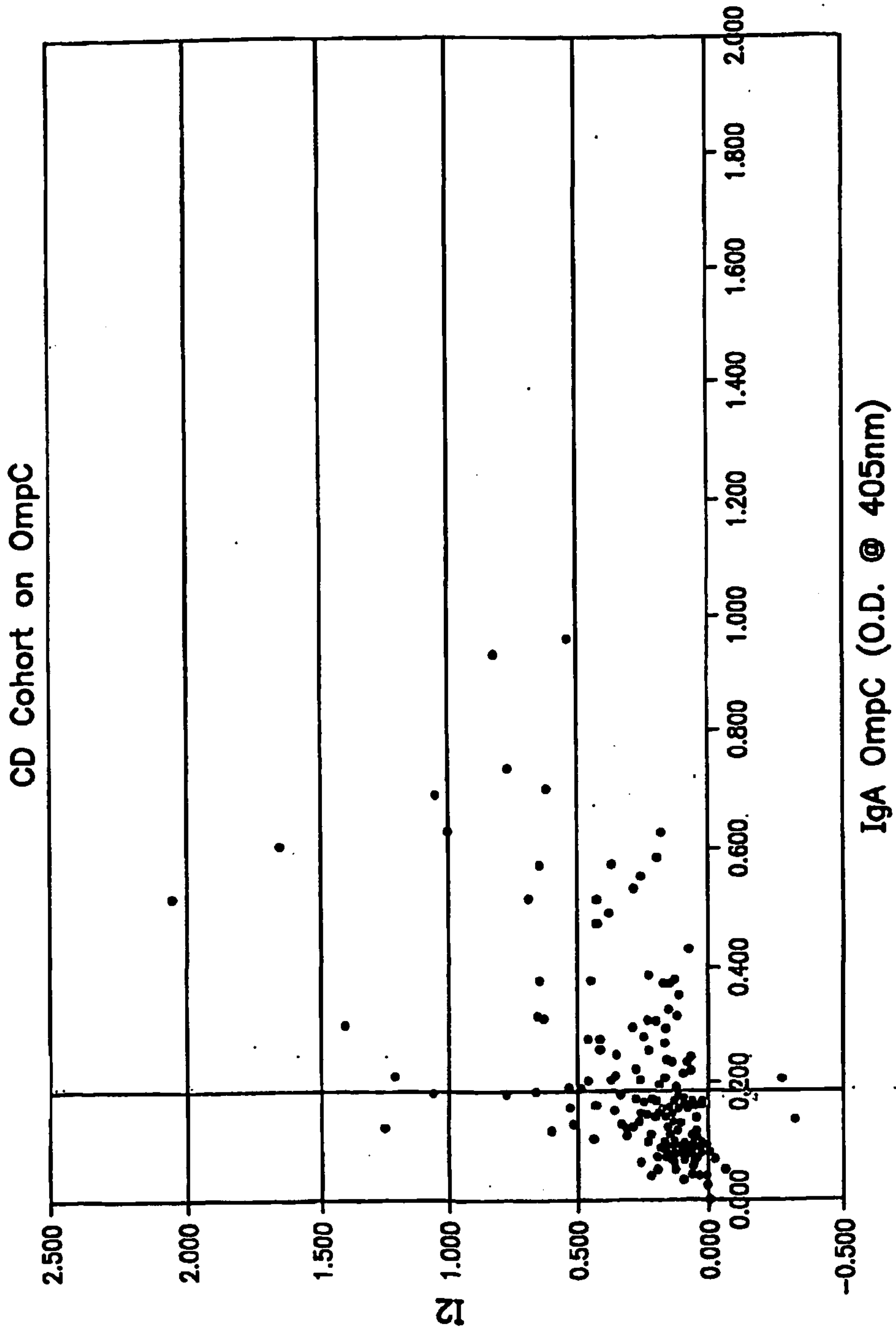
CD Cohort on OmpC
I2
IgA OmpC (O.D. @ 405nm)
2.500
2.000
1.500
1.000
0.500
0.000
-0.500
0.000
0.200
0.400
0.600
0.800
1.000
1.200
1.400
1.600
1.800
2.000

FIG. 1

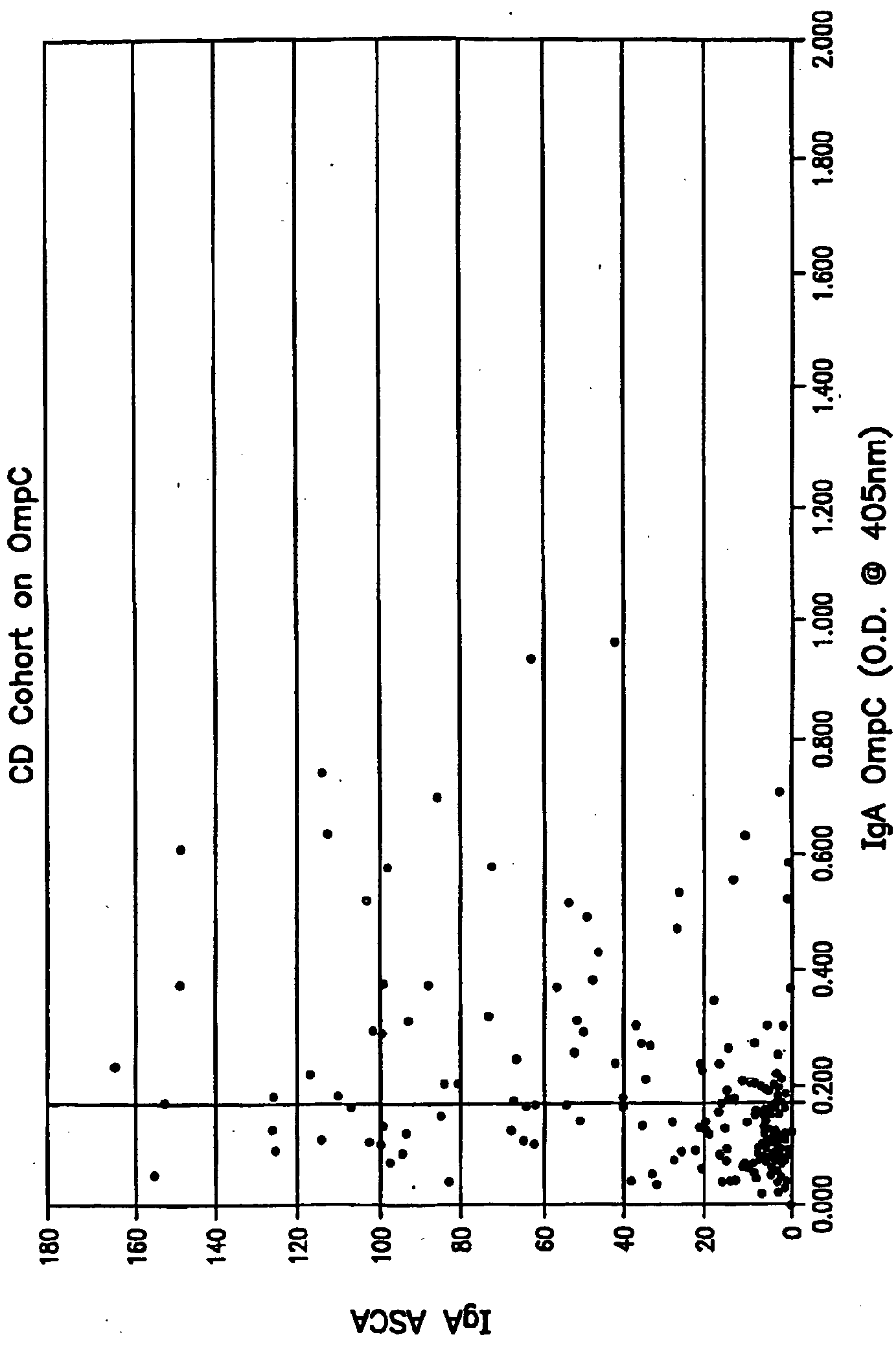
CD Cohort on OmpC
IgA ASCA
180
160
140
120
100
80
60
40
20
0
IgA OmpC (O.D. @ 405nm)
0.000
0.200
0.400
0.600
0.800
1.000
1.200
1.400
1.600
1.800
2.000

FIG. 2

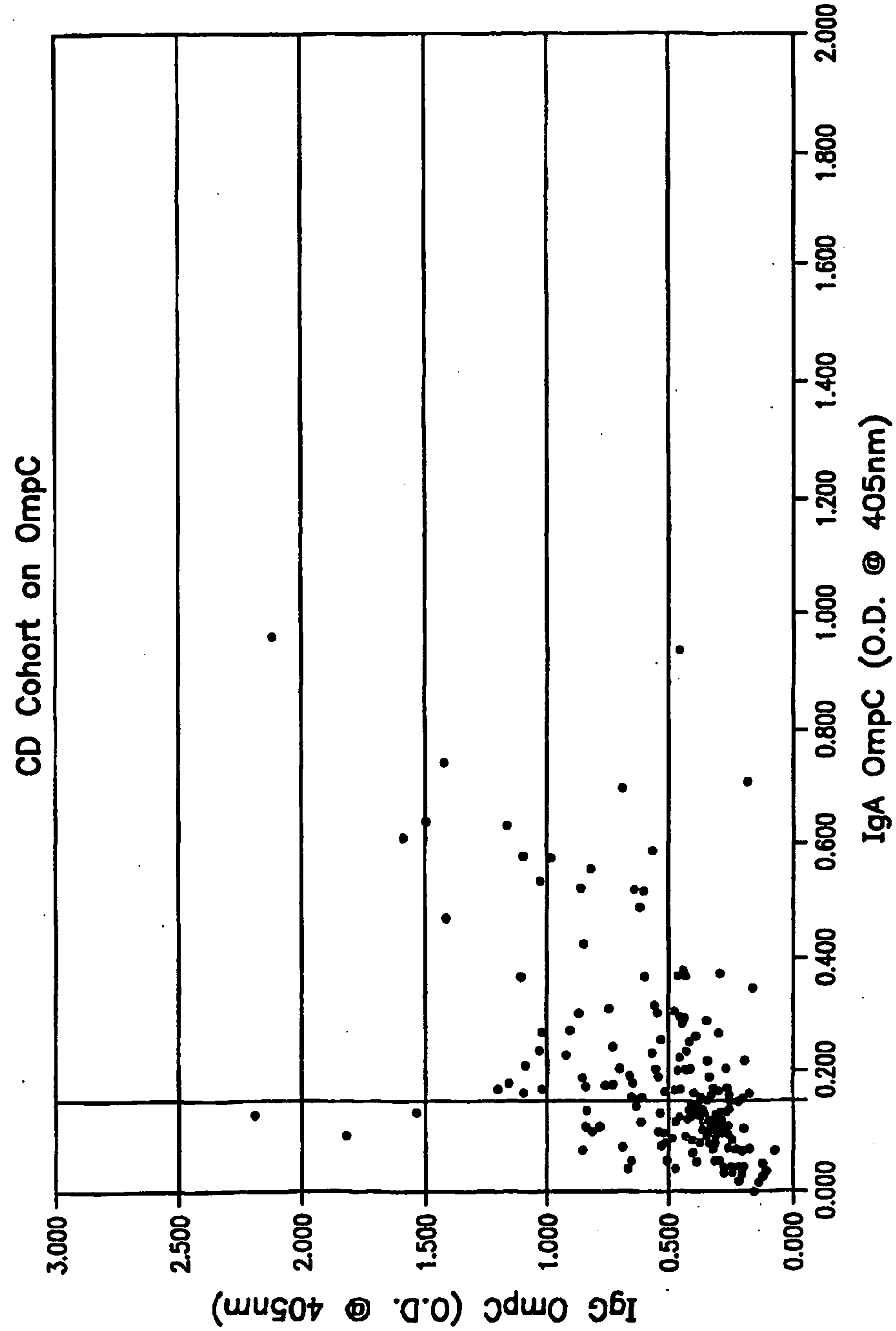
CD Cohort on OmpC
IgG OmpC (O.D. @ 405nm)
3.000
2.500
2.000
1.500
1.000
0.500
0.000
IgA OmpC (O.D. @ 405nm)
0.000
0.200
0.400
0.600
0.800
1.000
1.200
1.400
1.600
1.800
2.000

FIG. 3

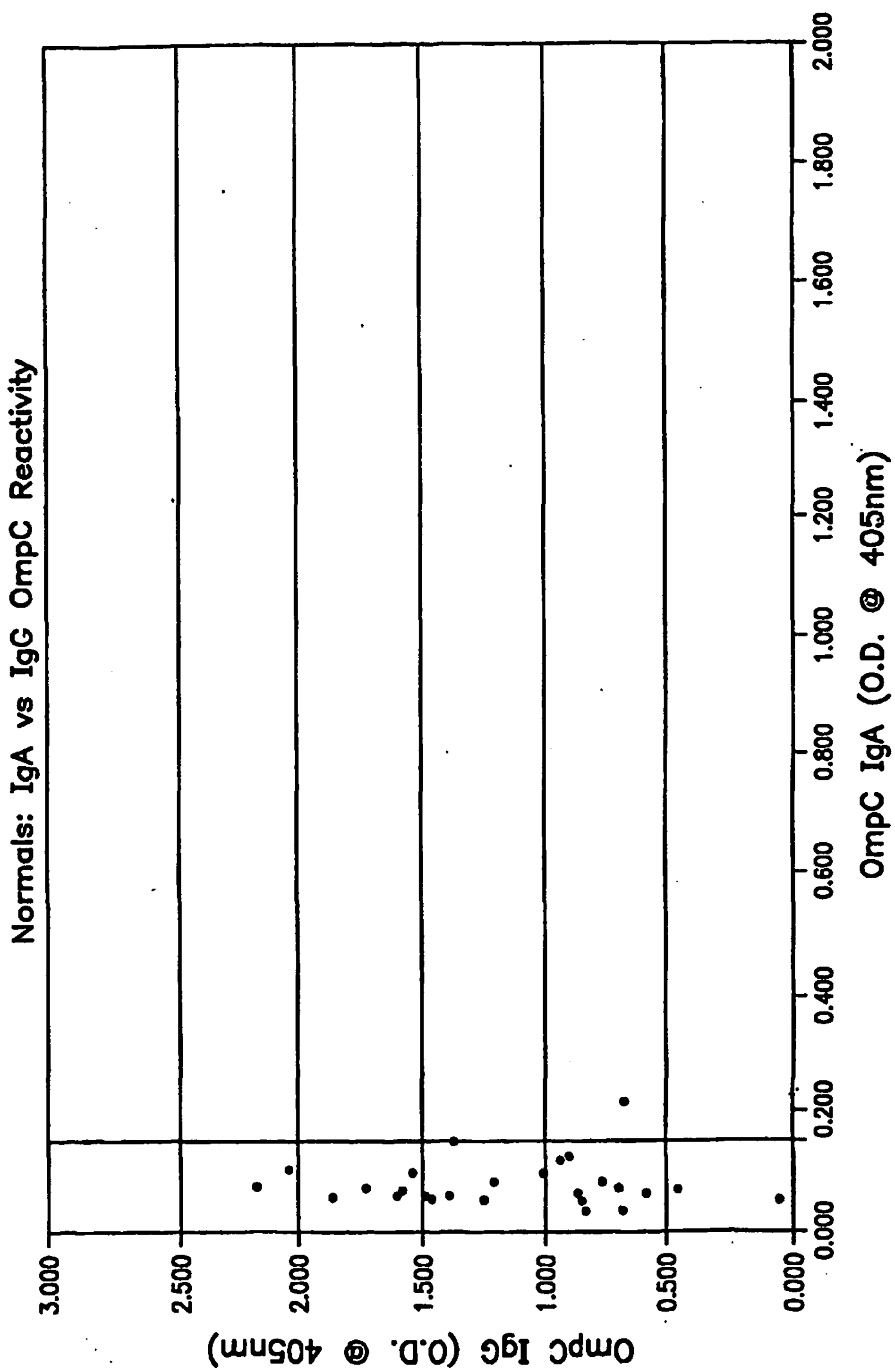
Normals: IgA vs IgG OmpC Reactivity
OmpC IgG (O.D. @ 405nm)
3.000
2.500
2.000
1.500
1.000
0.500
0.000
OmpC IgA (O.D. @ 405nm)
0.000
0.200
0.400
0.600
0.800
1.000
1.200
1.400
1.600
1.800
2.000

FIG. 4

```
E. coli 367 amino acids outer membrane protein c precursor:

  1 mkskvlalli pallaagaah aaevynkdgn kldlygkvdg lhyfsdnkdv dgdqtymrlg
     61 fkgetqvtdq ltgyggweyq iqgnsaenen nswtrvafag lkfqdvgsfd ygrnygvvyd
    121 vtswtdvlpe fggdtygsdn fmqqrgnfga tyrntdffgl vdglnfavqy qgkngnpsge
    181 gftsgvtnng rdalrqngdg vggsitydye gfgiggaiss skrtdaqnta ayigngdrae
    241 tytgglkyda nniylaaqyt qtynatrvgs lgwankaqnf eavaqyqfdf glrpslaylq
    301 skgknlgrgy ddedilkyvd vgatyyfnkn mstyvdykin llddnqftrd agintdniva
    361 lglvyqf
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FIG. 5

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A GAT CTG GCC AGC GCC GTG GGC ATC CAG TCC GGC AGC ATC TTT CAT CAC TTC AAG AGC AAG
 ▶ D   L   A   S   A   V   G   I   Q   S   G   S   I   F   H   H   F   K   S   K
 GAT GAG ATA TTG CGT GCC GTG ATG GAG GAA ACC ATC CAT TAC AAC ACC GCG ATG ATG CGC
▶ D   E   I   L   R   A   V   M   E   E   T   I   H   Y   N   T   A   M   M   R
 GCT TCA CTG GAG GAG GCG AGC ACG GTG CGC GAA CGC GTG CTG GCG CTG ATC CGC TGC GAG
▶ A   S   L   E   E   A   S   T   V   R   E   R   V   L   A   L   I   R   C   E
 TTG CAG TCG ATC ATG GGC GGC AGT GGC GAG GCC ATG GCG GTG CTG GTC TAC GAA TGG CGC
▶ L   Q   S   I   M   G   G   S   G   E   A   M   A   V   L   V   Y   E   W   R
 TCG CTG TCG GCC GAA GGC CAG GCG CAC GTG CTG GCC CTG CGT GAC GTG TAT GAG CAG ATC T
▶ S   L   S   A   E   G   Q   A   H   V   L   A   L   R   D   V   Y   E   Q   I
```

FIG. 6